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(54) Title: VECTORS FOR THE DIAGNOSIS AND TREATMENT OF SOLID TUMORS INCLUDING MELANOMA

(57) Abstract

The present invention is directed to the isolation and use of super-infective, tumor-specific vectors that are strains of parasites including, but not limited to, bacteria, fungi and protists. In certain embodiments, the parasites include, but are not limited to, the bacterium *Salmonella* spp., such as *Salmonella typhimurium*, the bacterium *Mycobacterium avium* and the protozoan *Leishmania amazonensis*. In other embodiments, the present invention is concerned with the isolation of super-infective, tumor-specific, suicide gene-containing strains of parasites for use in treatment of solid tumors.

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VECTORS FOR THE DIAGNOSIS AND TREATMENT OF SOLID TUMORS INCLUDING MELANOMA

This application is a continuation-in-part application of United States Patent Application Serial No. 08/486,422 filed June 7, 1995, which is incorporated by reference herein in its entirety.

1. FIELD OF THE INVENTION

The present invention is concerned with the isolation and use of super-infective, tumor-specific, attenuated strains of parasites including, but not limited to, bacteria, fungi and protists. In certain embodiments the parasites include the bacterium *Salmonella* spp., such as *Salmonella typhimurium*, the bacterium *Mycobacterium avium*, and the protozoan *Leishmania amazonensis*, for the diagnosis and treatment of sarcomas, carcinomas, and other solid tumor cancers. In other embodiments, the present invention is concerned with the isolation and use of super-infective, tumor-specific, suicide gene-containing strains of parasites.

2. BACKGROUND OF THE INVENTION

Citation or identification of any reference in Section 2 of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

A major problem in the chemotherapy of solid tumor cancers is the delivery of therapeutic agents, such as drugs, in sufficient concentrations to eradicate tumor cells while at the same time minimizing damage to normal cells. Thus, studies in many laboratories are directed toward the design of biological delivery systems, such as antibodies, cytokines, and viruses for targeted delivery of drugs, pro-drug converting enzymes, and/or genes into tumor cells. Houghton and Colt, 1993, New Perspectives in Cancer Diagnosis and Management 1: 65-70; de Palazzo,et al., 1992a, Cell. Immunol. 142:338-347; de Palazzo et al., 1992b, Cancer Res.

52: 5713-5719; Weiner, et al., 1993a, J. Immunotherapy 13:110-116; Weiner et al., 1993b, J. Immunol. 151:2877-2886; Adams et al., 1993, Cancer Res. 53:4026-4034; Fanger et al., 1990, FASEB J. 4:2846-2849; Fanger et al., 1991, Immunol. Today 12:51-54; Segal, et al., 1991, Ann N.Y. Acad. Sci. 636:288-294; Segal et al., 1992, Immunobiology 185:390-402; Wunderlich et al., 1992; Intl. J. Clin. Lab. Res. 22:17-20; George et al., 1994, J. Immunol. 152:1802-1811; Huston et al., 1993, Intl. Rev. Immunol. 10:195-217; Stafford et al., 1993, Cancer Res. 53:4026-4034; Haber et al., 1992, Ann. N.Y. Acad. Sci. 667:365-381; Haber, 1992, Ann. N.Y. Acad. Sci. 667: 365-381; Feloner and Rhodes, 1991, Nature 349:351-352; Sarver and Rossi, 1993, AIDS Research & Human Retroviruses 9:483-487; Levine and Friedmann, 1993, Am. J. Dis. Child 147:1167-1176; Friedmann, 1993, Mol. Genetic Med. 3:1-32; Gilboa and Smith, 1994, Trends in Genetics 10:139-144; Saito et al., 1994, Cancer Res. 54:3516-3520; Li et al., 1994, Blood 83:3403-3408; Vieweg et al., 1994, Cancer Res. 54:1760-1765; Lin et al., 1994, Science 265:666-669; Lu et al., 1994, Human Gene Therapy 5:203-208; Gansbacher et al., 1992, Blood 80:2817-2825; Gastl et al., 1992, Cancer Res. 52:6229-6236.

Because of their biospecificity, such systems could in theory deliver therapeutic agents to tumors. However, it has become apparent that numerous barriers exist in the delivery of therapeutic agents to solid tumors that may compromise the effectiveness of antibodies, cytokines, and viruses as delivery systems. Jain, 1994, Scientific American 7:58-65 (Jain). For example, in order for chemotherapeutic agents to eradicate metastatic tumor cells, they must

a) travel to the tumors via the vasculature;
b) extravasate from the small blood vessels supplying the tumor;
c) traverse through the tumor matrix to reach those tumor cells distal to the blood supply; and
d) interact effectively with the target tumor cells (adherence, invasion, pro-drug activation, etc).

Although antibodies and viruses can express specific recognition sites for tumor cells, they are dependent solely upon the forces of diffusion and convection in order to reach these sites. According to Jain:

> An agent that destroys cancers cells in a culture dish should, in theory, be able to kill such cells in the body.... Sadly, however, the existing pharmacopoeia has not markedly reduced the number of deaths caused by the most common solid tumors in adults, among them cancers of the lung, breast, colon, rectum, prostate, and brain.... Before a blood-borne drug can begin to attack malignant cells in a tumor, it must accomplish three critical tasks. It has to make its way into a microscopic blood vessel lying near malignant cells in the tumor, exit from the vessel into the surrounding matrix (the interstitium), and finally, migrate through the matrix to the cells. Unfortunately, tumors often develop in ways that hinder each of these steps.

Jain points out that blood vessels supplying tumors are irregular and convoluted in shape so that blood flow is frequently restricted compared to that in normally vascularized tissue. In addition, there is an unusually high interstitial pressure in many tumors that counteracts the blood flow. Jain further points out that the two chief forces governing the transport of agents to tumor cells via the circulatory system are convection (the transport of molecules by a stream of flowing fluid), and diffusion (the movement of molecules from an area of high concentration to an area of low concentration). Since tumors are often non-uniformly vascularized, many cells in the tumors receive nutrients through the process of diffusion through the

matrix. Jain and coworkers obtained data suggesting that "a continuously supplied monoclonal antibody having a molecular weight of 150,000 daltons could take several months to reach a uniform concentration in a tumor that measured one centimeter in radius and had no blood supply in its center."

2.1. BACTERIAL INFECTIONS AND CANCER

Regarding bacteria and cancer, an historical review reveals a number of clinical observations in which cancers were reported to regress in patients with bacterial infections. Nauts et al., 1953, Acta Medica. Scandinavica 145:1-102, (Suppl. 276) state:

> The treatment of cancer by injections of bacterial products is based on the fact that for over two hundred years neoplasms have been observed to regress following acute infections, principally streptococcal. If these cases were not too far advanced and the infections were of sufficient severity or duration, the tumors completely disappeared and the patients remained free from recurrence.

Shear, 1950, J. A.M.A. 142:383-390 (Shear), observed that 75% of the spontaneous remissions in untreated leukemia in the Children's Hospital in Boston occurred following an acute episode of bacterial infection. Shear stated:

> Are pathogenic and non-pathogenic organisms one of Nature's controls of microscopic foci of malignant disease, and in making progress in the control of infectious diseases, are we removing one of Nature's controls of cancer?

Subsequent evidence from a number of research laboratories indicated that at least some of the anti-cancer effects are mediated through stimulation of the host immune system, resulting in enhanced immuno-rejection of the cancer cells. For example, release of the lipopolysaccharide (LPS)

endotoxin by Gram negative bacteria such as *Salmonella* triggers release of tumor necrosis factor, TNF, by cells of the host immune system, such as macrophages, Christ et al., 1995, Science 268:80-83. Elevated TNF levels in turn initiate a cascade of cytokine-mediated reactions which culminate in the death of tumor cells. In this regard, Carswell et al., 1975, Proc. Natl. Acad. Sci. USA 72:3666-3669, demonstrated that mice injected with bacillus Calmette-Guerin (BCG) have increased serum levels of TNF and that TNF-positive serum caused necrosis of the sarcoma Meth A and other transplanted tumors in mice. Further, Klimpel et al., 1990, J. Immunol. 145:711-717, showed that fibroblasts infected *in vitro* with *Shigella* or *Salmonella* had increased susceptibility to TNF.

As a result of such observations as described above, immunization of cancer patients with BCG injections is currently utilized in some cancer therapy protocols. See Sosnowski, 1994, Compr. Ther. 20:695-701; Barth and Morton, 1995, Cancer 75 (Suppl. 2):726-734; Friberg, 1993, Med. Oncol. Tumor. Pharmacother. 10:31-36 for reviews of BCG therapy.

2.2. PARASITES AND CANCER CELLS

Although the natural biospecificity and evolutionary adaptability of parasites has been recognized for some time and the use of their specialized systems as models for new therapeutic procedures has been suggested, there are few reports of, or proposals for, the actual use of parasites as vectors.

In this regard, Pidherney et al., 1993, Cancer Letters 72:91-98 (Pidherney et al.) and Alizadeh et al., 1994, Infect. Immun. 62:1298-1303 (Alizadeh et al.) have provided evidence that the pathogenic free-living amoeba, *Acanthamoeba castellani*, has tumorcidal capabilities toward human tumor cells, including melanoma, when added to tumor cells growing in culture or when injected directly into tumors in nude mice. Pidherney et al. conclude:

> The feasibility of utilizing the tumorcidal properties of pathogenic/free-living amoebae and their cell-free products in the treatment of drug-resistant or radio-resistant tumors warrants further investigation.

However, Pidherney et al. also point out that such pathogenic/free living amoebae can exist either as free-living organisms feeding on bacteria or as opportunistic pathogens producing life-threatening meningoencephalitis or blinding keratitis.

Thus, it is readily apparent that for any parasite to be effective as a therapeutic vector, for example, for human tumors, the benefit of the parasite as a vector must outweigh its risk as a pathogen to the patient. Therefore, although Pidherney et al. and Alizadeh et al. demonstrated cytotoxicity of pathogenic amoebae toward tumor cells, and further suggested their use in the treatment of drug-resistant and radio-resistant tumors, they offered no solution for the inherent pathogenicity of these organisms once injected into cancer patients. Furthermore, they offered no method, *e.g.*, genetic selection for isolating super-infective, tumor-specific strains of pathogenic amoebae nor did they suggest insertion into the amoebael genome of genetic constructs containing inducible genes for the synthesis and secretion of pro-drug converting enzymes and/or suicide gene products.

Likewise, Lee et al., 1992, Proc. Natl. Acad. Sci. USA 89:1847-1851 (Lee et al.) and Jones et al., 1992, Infect. Immun. 60:2475-2480 (Jones et al.) isolated mutants of *Salmonella typhimurium* that were able to invade HEp-2 (human epidermoid carcinoma) cells *in vitro* in significantly greater numbers than the wild type strain. The "hyperinvasive" mutants were isolated under conditions of aerobic growth of the bacteria that normally repress the ability of wild type strains to invade HEp-2 animal cells. However, Lee et al. and Jones et al. did not suggest the use of such mutants as

therapeutic vectors, nor did they suggest the isolation of tumor-specific bacteria by selecting for mutants that show infection preference for melanoma or other cancers over normal cells of the body. Without tumor-specificity or other forms of attenuation, such hyperinvasive *Salmonella typhimurium* as described by Lee et al. and Jones et al. would likely be pan-invasive, causing wide-spread infection in the cancer patient. Further, without selection for tumor specificity or employment of other forms of attenuation, use of such bacteria as therapeutic vectors would increase the risk of pan-infection and septic shock to the cancer patient.

Pan et al., 1995, Nature Medicine 1:471-477 (Pan et al.) described the use of *Listeria monocytogenes* as a vaccine for the immunization of mice against lethal challenges with tumor cells expressing the same antigen expressed by the Listeria vaccine. In addition, they showed regression of established tumors when immunized after tumor development in an antigen specific T-cell-dependent manner. However, Pan et al. did not show that *Listeria monocytogenes* could be used as a tumor specific vector, which would target and amplify within the tumor. Rather, Pan et al. showed that recombinant *Listeria monocytogenes* has the ability to deliver a foreign antigen to the immune system and to involve cell-mediated immunity against the same antigen.

Sizemore et al., 1995, Science 270:299-302 (Sizemore et al.) described the use of attenuated *Shigella* bacteria as a DNA delivery vehicle for DNA-mediated immunization. Sizemore et al. showed that an attenuated strain of *Shigella* invaded mammalian cells in culture and delivered DNA plasmids containing foreign genes to the cytoplasm of the cells. Foreign protein was produced in the mammalian cells as a result of the procedure. The *Shigella* vector was designed to deliver DNA to colonic mucosa, providing a potential oral and mucosal DNA immunization procedure as well as other gene immunotherapy strategies. However, Sizemore et al. did not suggest the use of such attenuated *Shigella* as tumor vectors in that they could be

used to target tumors and thereby express genes within them. Rather, Sizemore et al. envisioned its use in vaccination therapy following oral delivery and invasion of the mucosa.

Clostridium was previously investigated as a potential therapeutic vector for solid tumors. The propensity of spores of the obligate anaerobe *Clostridium* to germinate in necrotic tissues is well known. Tetanus and gas gangrene result from successful colonization of necrotic tissue by pathogenic members of this genus.

Parker et al., 1947, Proc. Soc. Exp. Biol. Med. pp. 461-467 first showed that direct injection of spores of *Clostridium histolyticus* into a transplantable sarcoma growing in a mouse caused oncolysis, *i.e.*, liquification, as well as regression of the tumor. In general the process of *Clostridium*-mediated oncolysis was accompanied by acute toxicity and death of the mice. Malmgren and Flanigan, 1955, Cancer Res. 15:473 demonstrated that mice bearing mammary carcinomas, hepatomas, and other tumors died within 48 hrs of intravenous injection of *Clostridium tetani* spores, whereas control, non-tumor bearing animals were asymptomatic for 40 days. Möse and Möse, 1964, Cancer Res. 24:212-216 (Möse and Möse) described the colonization and oncolysis of tumors by *Clostridium butyricum*, strain M-55, a non-pathogenic soil isolate. Möse and Möse established the lack of human pathogenicity of the M-55 strain by administering spores to themselves, as reported by Carey et al., 1967, Eur. J. Cancer 3:37-46. Using *Clostridium butyricum* strain M-55, Möse and Möse reported that intravenous injections of spores caused oncolysis of the mouse Erlich ascites tumor, growing experimentally as a solid tumor. Aerobic spore-forming organisms--*e.g.*, *Bacillus mesentericus*, *Bacillus subtilis*, which were prepared in a similar manner, did not show any oncolysis under the same conditions. Möse and Möse concluded that the clostridial oncolysis was restricted to anaerobic areas of the tumors because of the anaerobic metabolic requirements of the bacteria.

Gericke and Engelbart, 1964, Cancer Res. 24:217-221 showed that intravenously injected spores of strain M-55 produced extensive lysis of a number of different tumors, but with shortened survival times of the *Clostridium*-treated, tumor-bearing animals compared to non-treated tumor-bearing animals. Further, they found that "metastases in organs or lymph nodes were unaffected by the spores unless the metastatic tumors had reached a considerable size."

Thiele et al., 1964, Cancer Res. 24:222-233 showed that intravenously injected spores of a number of species of nonpathogenic *Clostridia*, including M-55, localized and germinated in tumor tissue, but not in normal tissues of the mouse. Thiel et al., 1964, Cancer Res. 24:234-238 found that spore treatment produced no effect when administered early in the development of the tumor, *i.e.*, when the tumors were of small size. While the spores caused oncolysis in tumors of sufficient size, there was no effect in smaller tumors or metastases. The animals regularly died during oncolysis. Carey et al., 1967, Eur. J. Cancer 3:37-46, concluded that small tumors and metastases had been noted to be resistant to oncolysis whereas large neoplasms were particularly favorable. Thus, the qualitative differences in germination of spores were likely to be not a characteristic of neoplastic and normal tissues per se, but related to physiologic and biochemical conditions found within large tumor masses.

Recent molecular genetic studies have focused on anaerobic bacteria of the genus *Clostridium* as potential tumor vectors. Fox et al., 1996, Gene Therapy 3:173-178 using a *Clostridium* expression vector were able to transform the *E. coli* cytosine deaminase gene into *Clostridium beijerincki*, which resulted in increased cytosine deaminase activity in the growth medium supernatant and cell extracts of transformed clostridial bacteria. Such supernatants, when added to cultures of mouse EMT6 carcinoma made the cells sensitive to 5-fluorocytosine, presumably through its conversion to the toxic 5-fluorouracil. Similarly, Minton et

al., 1996, FEMS Microbiol. Rev. 17:357-364 inserted the *E. coli* nitroreductase gene into *Clostridium beijerincki* and were able to detect expression of the gene in an *in vivo* murine tumor model through the use of antibodies directed against the *E. coli* nitroreductase gene. The nitroreductase gene product activates CB1954, a potent alkylating agent.

Nothing in any of the above references (or any other references known to the present inventors) suggests the use of any microorganisms, other than the obligate anaerobe *Clostridium*, as a potential therapeutic vector for solid tumors.

2.3. **ATTENUATED *SALMONELLA* SPP.**

Bacon et al., 1950, Br. J. Exp. Path. 31:703-713; Br. J. Exp. Path. 31:714-724; 1951, Br. J. Exp. Path. 32:85-96 demonstrated that attenuation of *Salmonella* for virulence in mice can be achieved through auxotrophic mutations, *i.e.*, through the use of mutants which lack the ability to synthesize precursor molecules necessary for growth. More specifically, the authors showed that purine-requiring (Pur^-) auxotrophs of *Salmonella* were attenuated in mice.

Hoiseth and Stocker, 1981, Nature 291: 238-239 showed that *Salmonella typhimurium* auxotrophic mutants with requirements for aromatic amino acids (Aro^-) were attenuated for virulence in C57BL mice. Further, Su et al., 1992, Microbiol. Pathogenesis 13:465-476 showed that one such Aro^- mutant, the attenuated antigen carrier strain of *Salmonella typhimurium*, SL3261, was useful as a vaccine. The Shiga toxin B-subunit/hemolysin A (C-terminus) fusion protein was expressed and underwent extracellular export resulting in antigen-specific immune responses in mice inoculated with these bacteria.

O'Callaghan et al., 1988, Infect. Immun. 56:419-423 characterized *Salmonella typhimurium* that were both Aro- and Pur- and found that although they were highly attenuated in BALB/c mice, they persisted for several weeks in the livers and spleens following i.v. injections. They were found to be

ineffective as vaccines when administered either orally or i.v.

Johnson et al., 1991, Mol. Microbiol. 5:401-407 (Johnson et al.) demonstrated that attenuation in *Salmonella* virulence can be achieved through mutations in the heat shock inducible protein HtrA, a serine protease. Chabalgoity et al., 1996, Mol. Microbiol. 19:791-801, demonstrated that such attenuated htrA- *Salmonella typhimurium* were useful as live vaccines.

However, none of the references by Bacon et al., Hoiseth and Stocker, O'Callaghan et al., Johnson et al., Su et al. 1992, Chabalgoity et al. 1996, nor any of the studies referred to in Table 4, *infra*, suggest that such avirulent strains of *Salmonella typhimurium* would survive and proliferate within solid tumors, nor that such avirulent mutants might be used as vectors for solid tumor therapy.

2.4. OBJECTIVES OF THE INVENTION

The problems associated with the many physical barriers for delivery of therapeutic agents to solid tumors provide clear and difficult obstacles in the design of effective delivery systems. Thus, there has been a long felt need in the art to provide delivery systems which are able to overcome these obstacles.

It is an object of the present invention to use and to provide more advanced biological vectors such as parasites having several distinct advantages as a novel delivery system, some of which are listed below, as well as to meet the challenges of tumor therapy.

Antibiotic Sensitivity: It is an advantage for a tumor-specific parasitic vector to be sensitive to exogenously administered antibiotics. Parasites, such as bacteria, can be eradicated within their hosts by the administration of antibiotics. Such antibiotic sensitivity allows for the eradication of the parasite from the cancer patient's body upon completion of the therapeutic protocol.

Biospecificity: It is an advantage for a vector to express specificity for its target cell, *e.g.*, a tumor cell. The more specificity, of the vector for the tumor cell, the lower the inoculum necessary for effective therapy, thereby reducing the risk of septic shock or pan-infection to the cancer patient. Parasites show a great degree of natural biospecificity, having evolved to utilize a variety of specific recognition and invasion mechanisms. (For general discussions on biospecificity see: Falkow, 1991, Cell 65:1099-1102; Tumomanen, 1993, Am. Soc. Microbiol. 59:292-296).

Mutant Isolation and Genetic Manipulation: It is an advantage, in the design and isolation of a parasite as a tumor-specific, therapeutic vector, for the parasite to be amenable to genetic manipulation. Parasites with haploid genomes and short generation times, for example, bacteria such as *Salmonella typhimurium* and enteroinvasive *Escherichia coli*, can be readily subjected to mutagenesis followed by enrichment procedures for the isolation of strains with desired new characteristics (see generally, Neidhardt et al., (ed.) 1987, *Escherichia coli* and *Salmonella typhimurium*, Cellular and Molecular Biology. American Society for Microbiology, pp 990-1033. Furthermore, the methods for the genetic analysis and stable introduction of genetic constructs into these bacteria are well known to the science of molecular genetics.

Chemotaxis: A chemotactic response toward cancer cells is an advantage for a tumor-specific vector, for example as a stimulus for the vector to invade through a basement membrane matrix such as that produced by endothelial cells in the vasculature, or as a stimulus for the vector to seek out cancer cells surrounded by tumor matrix. Chemotactic responses in parasites and commensalists or mutualists, particularly in bacteria such as *Escherichia coli* and *Salmonella typhimurium*, are well documented. For a review of chemotaxis see Macnab, 1992, Ann. Rev. Genet. 26:131-158.

Replication Within Target Cells: The ability to replicate within target cells is an advantage for a tumor-specific vector. Such an ability allows for amplification of the therapeutic vector number within the infected cancer cell, thus increasing the therapeutic effectiveness of the vector. Progeny of vectors within cancer cells further infect surrounding or distant cancer cells, thus amplifying the vector number within the tumor cell population.

Anaerobic and Aerobic Metabolism: The ability to express invasive and amplification capacities under either aerobic or anaerobic conditions is an advantage for a tumor-specific vector. Solid tumors generally contain vascularized, oxygen-rich areas as well as necrotic oxygen-poor areas. A vector that is functional in both such environments would be able to reach a larger portion of tumor cells than one that can function in only one environment, such as, for example, an obligate anaerobe or aerobe.

3. SUMMARY OF THE INVENTION

The present invention provides compositions and methods for delivery of genes and/or gene products to and/or into target mammalian cells *in vitro* or *in vivo*. The genes and/or gene products are delivered by microorganism vectors, including bacteria, fungal and protozoan parasites, which are selected and/or genetically engineered to be specific to a particular type of target mammalian cell. In a preferred embodiment, the vectors function under both aerobic and anaerobic conditions, are super-infective, tumor-specific microorganisms useful for diagnosis or treatment of sarcomas, carcinomas, lymphomas or other solid tumor cancers, such as germ line tumors and tumors of the central nervous system, including, but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma.

Vectors useful for the methods of the present invention include but are not limited to *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Streptococcus* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, BCG, *Mycoplasma hominis*, *Rickettsiae quintana*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp., *Trypanasoma cruzi*, *Encephahtozoon cuniculi*, *Nosema helminthorum*, *Unikaryon legeri*.

As used herein, *Salmonella typhimurium* encompasses all *Salmonella* species. It has long been recognized that the various "species" of the genus *Salmonella* are in fact a single species by all acceptable criteria of bacterial taxonomy. The single species is now designated "*Salmonella enterica*". F. Neidhardt (ed.), *Escherichia coli* and *Salmonella*, 1996, Volume I, pp. *xx*, ASM Press, Washington DC.

An embodiment of the present invention is to provide methods for the isolation of super-infective, attenuated, tumor-specific mutants of microorganisms such as bacterial, fungal and protozoan parasites. Further, the present invention provides methods for use of these microorganisms in the diagnosis and treatment of malignant and/or metastatic solid tumor cancers, such as melanoma or colon cancer. Moreover, these mutant parasites may express specific gene products, some of which are secreted into the cytoplasm or vacuolar space of the infected cell.

The present invention provides methods for the isolation of super-infective target cell-specific microorganisms. In particular embodiments, the invention provides for the isolation and use of super-infective, tumor-

specific strains of parasites such as the bacterium *Salmonella* spp., including *S. typhimurium*, the bacterium *Mycobacterium avium*, and the protozoan *Leishmania amazonensis*. The tumor-specific vectors can also contain suicide genes.

One embodiment of the present invention provides methods for the isolation of and compositions comprising super-infective, tumor-specific mutants of *Salmonella* spp., *e.g.*, *Salmonella typhimurium*, and for their use in the diagnosis and treatment of sarcomas, carcinomas, melanomas, colon cancer, and other solid tumor cancers. Another embodiment of the present invention provides methods for the isolation of and compositions comprising super-infective, tumor-specific mutants of *Salmonella* spp. containing a suicide gene. In a specific embodiment, the suicide gene is thymidine kinase from *Herpes simplex* virus or cytosine deaminase from *Escherichia coli* or human microsomal p450 oxidoreductase.

Another embodiment of the present invention provides methods for the isolation of and compositions comprising super-infective, tumor-specific mutants of the protozoan, *Leishmania amazonensis* and for their use in the diagnosis and treatment of sarcomas, carcinomas, melanomas, colon cancer, and other solid tumor cancers.

Yet another embodiment of the present invention provides methods for the isolation of and compositions comprising super-infective, tumor-specific mutants of the bacterium *Mycobacterium avium* and for their use in the diagnosis and treatment of sarcomas, carcinomas, melanomas, colon cancer, and other solid tumor cancers.

Yet another embodiment of the present invention provides methods for attenuation of parasite vector toxicity so as to reduce the risk of septic shock or other complications in the host, *i.e.*, the patient receiving vector-delivered gene therapy. Such methods include mutagenesis of parasites; isolation of parasite mutants with increased tumor specificity, increased specificity for

suicide gene expression and concomitant reduced ability to infect normal host cells in the body; isolation of mutants with enhanced chemotactic abilities toward cancer cell secretory products; isolation of mutants with genetically altered lipopolysaccharide composition; and isolation of mutants with altered virulence genes so as to achieve specific survival of the parasitic vector in cancer cells as opposed to normal cells of the host body.

The present invention further encompasses use of microorganism vectors for diagnosis or treatment of solid tumor cancers.

The present invention may be understood more fully by reference to the following definitions, detailed description of the invention, illustrative examples of specific embodiments and the appended figures in which:

4. **DEFINITIONS**

Attenuation:	Attenuation, in addition to its traditional definition in which a microorganism or vector is modified so that the microorganism or vector is less pathogenic, is intended to include also the modification of a microorganism or vector so that a lower titer of that microorganism or vector can be administered to a patient and still achieve comparable results as if one had administered a higher titer of the parental microorganism or vector. The end result of attenuation is that the risk of toxicity as well as other side-effects is decreased, when the microorganism or vector is administered to the patient.
Suicide gene:	A suicide gene is defined as a gene that when delivered to a target cell

and expressed by a vector of the present invention causes the death of the target cell and/or the vector.

Super-infective: A super-infective vector is defined as a vector which is able to attach and/or infect a target cell more readily as compared to the wild type vector. Depending on the population density of the inoculum, the ratio between super-infective vectors and wild type vectors detectably infecting a target cell approaches 4:1, preferably 30:1, more preferably 90:1. Most preferably, one is able to reduce the inoculum size and infection time so that only the super-infective vectors have time to attach to and/or infect cancer cells growing in cell culture *in vitro* or as tumors *in vivo*.

Tumor-specific: A tumor-specific vector is defined as a vector which is able to distinguish between a cancerous target cell and the non-cancerous counterpart cell so that the vector preferentially attaches to, infects and/or remains viable in the cancerous target cell.

5. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Figure 1 depicts a DNA cassette system for expressing pro-drug converting enzymes. Each of the components is generated by PCR using primers containing specific restriction endonuclease sites *NotI*, *NsiI*, *NcoI*, *SfiI* or *PacI* that allow for simple interchange of individual components. For example, (A) is the coding sequence for pro-

drug converting enzymes such as thymidine kinase, cytosine deaminase or human microsomal p450 oxidoreductase; (B) is a promoter, which is active in an inducible, constitutive or cell specific manner; (C) is a N-terminal secretion signal sequence, such as the β-lactamase signal sequence; and (D) is a C-terminal secretion signal sequence, such as the enteroinvasive *E. coli* hemolysin A signal sequence.

Figure 2A-B. Figure 2A-B are photomicrographs of *Salmonella typhimurium* wild type strain ATCC No. 14028 infecting human melanoma cell line M2. A starting population of ATCC No. 14028 was subjected to 10 cycles of infection into and recovery from M2 melanoma cells before use in the infection assay shown in Figure 2A-B. Figure 2A. Light micrograph of an infected melanoma cell. Figure 2B. DAPI staining of the cell showing cell nucleus, (n), and numerous bacteria inside the cell, (arrow).

Figure 3A-C. Figure 3A-C are photomicrographs of *Salmonella typhimurium* wild type strain ATCC No. 14028 during the process of internalization into human melanoma cell line M2. Figure 3A. Phase contrast micrograph of a host cell. Figure 3B. DAPI staining of the host cell showing the position of the bacteria, (arrow), and the host cell nucleus, (n). Figure 3C. Lysosomal glycoprotein LAMP-1 antibody staining of the host cell showing co-localization of the bacteria with lysosomes and/or melanosomes.

Figure 4A-4F relate to producing converting enzyme expression constructs and expression using the same.

Figure 4A-B. Expression of the *Herpes simplex* thymidine kinase gene containing a β-lactamase secretory signal sequence in *Salmonella typhimurium* super-infective clone 72. Figure 4A. Immunoblot analysis of *Salmonella typhimurium* strains using an anti-TK monoclonal antibody. Lane 1: bacteria containing only the plasmid vector p279; Lane 2: strain 14028 wt (CDC6516-60) (MO) containing the cytoplasmicly expressed form of TK (pHETK2); Lane 3: strain 14028 clone 72 containing the cytoplasmicly expressed form of TK (pHETK2); Lane 4: strain 14028 wt (MO) containing the β-

lactamase fusion form of TK (p5-3); Lane 5: strain 14028 clone 72 containing the β-lactamase fusion form of TK (p5-3); Lane 6: strain 14028 wt (MO) containing the β-lactamase fusion form of TK (p21A-2); Lane 7: strain 14028 clone 72 containing the β-lactamase fusion form of TK (p21A-2). Relative molecular mass x 10^3 is shown on the left. No antibody reactivity is seen in the "vector only" control (Lane 1). In each lane where the cytoplasmically expressed TK is present (Lanes 2 and 3) two major isoforms of the protein are seen; a higher molecular mass isoform containing a leader sequence and a lower molecular mass isoform wherein the leader sequence has been proteolytically cleaved off. In each lane where the bacteria express the TK gene β-lactamase signal sequence fusion (Lanes 4 to 7) two major isoforms of the protein are also seen: a higher molecular mass form containing the signal sequence and a lower molecular mass isoform wherein the signal sequence has been proteolytically cleaved off, which is the same apparent molecular mass as the processed form of the cytoplasmic enzyme.

Figure 4B. Relative TK enzyme activity associated with each of the samples in Figure A. Enzyme activity is expressed as the total number of counts of ^{125}IdC phosphorylated in a standard assay, Summers and Summers, 1977, J. Virol. 24:314-318. A small background is present in a bacterial extract from the vector only sample (Lane 1). Significantly higher levels of TK activity are observed in the wild type and the super-infective clone 72 containing the cytoplasmic form of TK pHETK2, Lanes 2 and 3. Similar levels are observed in both the wild type and super-infective clone 72 containing the β-lactamase signal sequence fusion isoform of TK p5-3, Lanes 4 and 5. Lower levels are observed in both the wild type and super-infective clone 72 containing the β-lactamase signal sequence fusion isoform of TK p21A-2, Lanes 6 and 7.

Figure 4-C is a schematic of the different *Herpes Simplex Virus* thymidine kinase secretion and expression constructs.

Figure 4-D is a schematic of the different human microsomal cytochrome p450 oxidoreductase expression constructs.

Figure 4-E is a schematic of the *E. coli* cytosine deaminase secretion and expression construct.

Figure 4-F is a graph showing the amount of 5-FC converted to 5-FU by different bacteria.

Figure 5A-B. Figure 5A-B are photomicrographs of histologic sections from a Cloudman S91 melanoma/macrophage hybrid #48 growing subcutaneously in a DBA/2J mouse. The tumor was excised from a mouse that had been inoculated 2 days earlier with 3 x 10^5 c.f.u. *Salmonella typhimurium* super-infective clone #72 carrying the *HSV* TK gene, clone #72^{5-3-2}. Figure 5A. A section stained with hematoxylin and eosin shows tumor cells with a central area of necrosis, denoted by arrows. Figure 5B. A section stained with Brown-Brenn stain (tissue gram stain) shows gram negative bacteria in a necrotic area of the tumor, denoted by the arrow. When viewed under a light microscope, the bacteria stain pink/purple against a yellow background.

Figure 6. Figure 6 is an electron micrograph of a section of a Cloudman S91 melanoma/macrophage hybrid #48 tumor excised from a DBA/2J mouse that had been inoculated i.p. 42 hours earlier with 4 x 10^6 *Salmonella typhimurium* super-infective clone 72. Visible in the micrograph are two *Salmonella typhimurium* bacteria, denoted by arrows, along with numerous melanosomes (m), sub-cellular organelles characteristic of melanoma cells. The co-localization of *Salmonella* and such melanosomes indicates that the bacteria are present in the cytoplasm of the melanoma cell. Magnification=21,000x.

Figure 7. Figure 7 is a photomicrograph of a histologic section from a B16F10 melanoma growing subcutaneously in a C57BL/6J mouse. The tumor was excised from a mouse that had been inoculated 42 hours earlier with 1.8 x 10^5 c.f.u. *Salmonella typhimurium* super-infective clone #72 carrying the *HSV* TK gene. The sections were from the

same tumor examined with the electron microscope as detailed in Figure 8. The section was stained with Brown-Brenn stain (tissue gram stain) and shows gram negative bacteria in a necrotic area of the tumor, denoted by arrows. When viewed under a light microscope, the bacteria stain pink/purple against a yellow background.

Figure 8. Figure 8 is an electron micrograph of a section from a B16F10 melanoma tumor excised from a C57BL/6J mouse that had been inoculated i.p. 42 hours earlier with 1.8 x 10^5 *Salmonella typhimurium* super-infective clone #72 carrying the *HSV* TK gene. The section was from the same tumor examined with the light microscope as detailed in Figure 7. The micrograph shows numerous *Salmonella typhimurium* in extracellular spaces, denoted by arrows, and in an area of necrosis. A single bacterium is also seen within the cytoplasm of a dying melanoma cell. The cytoplasm of the dying melanoma cell also contains numerous black melanosomes (m), characteristic of the B16F10 melanoma. Magnification = 9,750x.

Figure 9A-D. Figures 9A-D depict growth of Cloudman S91 melanoma/macrophage hybrid #48 tumors in DBA/2J mice under various treatment conditions. Mice were inoculated s.c. in the flank region with 3 x 10^5 melanoma cells. The tumors were palpable 8-10 days later, and some of the mice were then further inoculated with *Salmonella typhimurium* super-infective clone #72 carrying the *HSV* TK gene. Twenty-four hours post inoculation with bacteria, some groups of the mice were further inoculated i.p. with 2.0 mg ganciclovir. Ganciclovir inoculations were repeated 6 times over a 5 day period. Points represent caliper measurements of tumors in 2 to 5 mice per treatment group at the days indicated. Measurements in mm were made of length, width, and height for each tumor and volumes were calculated in mm^3. Average tumor volumes for each group of mice were defined as 100% on day 0, the beginning day of treatment. (Figure 9A). Control mice: no *Salmonella*; no ganciclovir (Figure 9B).

Ganciclovir only; (Figure 9C). *Salmonella* only; (Figure 9D). *Salmonella* + ganciclovir.

Figure 10A-B. Figure 10A shows a control mouse and Figure 10B shows a *Salmonella typhimurium*-infected (7B) DBA/2J mouse. The mice were inoculated (s.c.) with Cloudman S91 melanoma/macrophage hybrid #48 tumor cells. Upon the appearance of palpable tumors some of the mice were inoculated (i.p.) with 3 x 10^5 c.f.u. *Salmonella typhimurium* clone 72 containing the *HSV* TK gene (clone #72^{5-3-2}), allowed to eat and drink ad libitum for 10 days, and then treated with Sulfatrim™ antibiotic in their drinking water for several more days and photographed. Tumors in the depicted mice were representative of the general state of tumor progression in mice in *Salmonella*-treated and untreated cages.

Figure 11A-11H relate to the effects of gancicylovir on tumor cell growth, *in vivo* or *in vitro*.

Figure 11A-B. Figures 11A-B depict a control (Figure 11A) and a *Salmonella typhimurium*-infected (Figure 11B) DBA/2J mouse. The mice were inoculated (s.c.) with Cloudman S91 melanoma/macrophage hybrid #48 tumor cells. Upon the appearance of palpable tumors, some of the mice were inoculated with 3 x 10^5 c.f.u. *Salmonella typhimurium* clone #72 containing the *HSV* TK gene (clone #72^{5-3-2}). Control and Salmonella-infected mice were then injected (i.p.) with 2.0 mg ganciclovir a total of 5 times over a 4 day period. The mice were then treated with Sulfatrim™ antibiotic in their drinking water for several more days and photographed. The depicted mice are representative of the general state of tumor progression in mice, either in *Salmonella*-treated and untreated cages.

Figures 11(C-E) show the effect of ganciclovir on the growth of B16F10 melanomas in mice with and without inoculation of *Salmonella typhimurium* clone YS7211 (Figure 11-1 A); clone YS7213 (Figure 11-1 B); and clone YS7212 (Figure 11-1 C).

Figure 11-F is a graph showing the growth of B16F10 melanoma cells in monolayer culture in the presence or absence of ganciclovir at 10 μg/ml or 25 μg/ml.

Figure 11-G is a graph showing the effect of ganciclovir on the growth of B16F10 melanomas in mice following inoculation of *Salmonella typhimurium* clone YS7211 carrying the *HSV* thymidine kinase gene, YS7211/p5-3, with and without ganciclovir.

Figure 11-H is a graph showing the effect of total amounts of ganciclovir on the growth of B16F10 melanomas in mice following inoculation with *Salmonella typhimurium* clone YS7211 carrying the *HSV* thymidine kinase gene, YS7211/p5-3.

Figure 12A-B are electron micrographs. Figure 12A is an electron micrograph of a section from a HCT 116 human colon tumor excised from a BALB/c *nu/nu* mouse. The mouse had been inoculated i.p. 72 hours earlier with 2.8×10^5 c.f u. *Salmonella typhimurium* super-infective clone #72 containing the *HSV* TK gene, clone #72^{5-3-2}. Shown in the micrograph are numerous *Salmonella typhimurium* within the cytoplasm of a neutrophil associated with the tumor. Some of the bacteria are undergoing division as denoted by arrows. The neutrophil or polymorphonucleoleukocyte is characterized by its multi-lobed nucleus (n), (Magnification=21,000x).

Figure 12-B is an electron micrograph showing numerous *Salmonella typhimurium*, denoted by arrows, in extracellular spaces as well as contained within a single cell, possibly a neutrophil, seen in the upper left. Also seen in the field are two unidentified cells that appear to be dying as indicated by the large intracellular space, along with cellular debris.

Figure 13. Figures 13A-B depict *Leishmania amazonensis* adhesion to human melanoma cell line M2. Figure 13A. Phase contrast micrograph showing parasites attached to cell, (arrow). Figure 13B. DAPI staining showing the parasite DNA, (arrows), and the host cell nucleus, (n).

Figure 14. Figures 14A-C are photomicrographs of *Leishmania amazonensis* during the process of internalization

into human melanoma cell line M2. Figure 14A. Phase contrast of a *Leishmania* trypomastigote, (arrow), entering a host cell. Figure 14B. DAPI staining showing the position of the parasite, (arrow), and the host cell nucleus, (n). Figure 14C. Lysosomal glycoprotein LAMP-1 antibody staining of the host cell showing co-localization of the bacteria and the lysosomes.

Figure 15 A-D. Figure 15 A-C are graphs showing growth of *Salmonella typhimurium* clone 72 and clone YS7212 in minimal Medium 56 supplemented with glucose only; Medium 56 with glucose plus adenine, vitamin B1, isoleucine, valine, and uracil; or Medium 56 with tumor extract (10%) only.

Figure 15-D is a graph depicting growth of *Salmonella typhimurium* clones 72 and YS7212 following invasion into human M2 melanoma cells in culture.

Figure 16 A-D are graphs showing growth of B16F10 melanomas in C57B6 mice with and without inoculation of *Salmonella typhimurium* strains YS721 (Figure 16-A); YS7213 (Figure 16-B); YS7211 (Figure 16-C); and YS7212 (Figure 16-D).

Figure 17 is a graph showing that combination of CD and 5-fluorocytosine prolong the survival of animals bearing B16F10 lung metastases when the animals are infected with a tumor-specific vector carrying the cytosine deaminase expression construct YS7212/pCD-Sec1.

Figure 18 is a graph showing TNF-α production by human macrophages incubated with lipopolysaccharide isolated from wild type and attenuated strains of *Salmonella typhimurium*.

Figure 19 is a bar graph demonstrating the effect of clones YS7211 and YS7212 expressing the *HSV* thymidine kinase gene, YS7211/p5-3 and YS7212/p5-3, respectively, on mice bearing metastatic B16F10 tumors with or without ganciclovir treatment.

6. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the isolation of novel therapeutic and diagnostic parasitic vectors for solid tumor cancers, such as sarcomas, carcinomas, lymphomas or other solid tumor cancers, for example, germ line tumors and tumors of the central nervous system, including, but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, melanoma and their use. Described, in detail below, are the novel intracellular parasite vectors; methods for the isolation of the novel vectors; genetic engineering of the isolated vectors; and methods for use of the novel vectors as well as other vectors in treatment or detection of solid malignant tumors, including metastatic tumors and tumor cells.

6.1. NOVEL VECTORS AND METHODS FOR THEIR ISOLATION

The isolated vectors, which are for example, bacteria, fungi or protista, are able to differentiate between cancerous cells and non-cancerous counterpart cells. For example, the isolated vectors are able to differentiate melanoma cells from melanocytes or differentiate colon cancer cells from normal colon epithelial cells. Table I is a representative list, which is in no way meant to limit the present invention, of intracellular parasitic and pathogenic microorganisms which are useful as tumor-specific vectors for the present invention and/or for isolation of novel mutant strains which are super-infective and tumor-specific vectors for use in the present invention.

TABLE 1

REPRESENTATIVE LIST OF ORGANISMS USEFUL AS VECTORS

Gram negative bacteria
Borrelia burgdorferi
Brucella melitensis
Escherichia coli
enteroinvasive *Escherichia coli*
Legionella pneumophila
Salmonella typhi
Salmonella typhimurium
Shigella spp.
Treponema pallidum
Yersinia enterocohtica

Gram positive bacteria
BCG (Bacillus Calmette-Guerin)
Chlamydia trachomatis
Listeria monocytogenies
Mycobacterium avium
Mycobacterium bovis
Mycobacterium tuberculosis
Mycoplasma hominis
Rickettsiae quintana
Streptococcus spp.

Fungi
Cryptococcus neoformans
Histoplasma capsulatum
Pneumocystis carnii

Apicomplexans
Eimeria acervulina
Neospora caninum
Plasmodium falciparum
Sarcocystis suihominis
Toxoplasma gondii

Kinetoplastida
Leishmania amazonensis
Leishmania major
Leishmania mexacana
Leptomonas karyophilus
Phytomonas spp.
Trypanasoma cruzi

Microsporidians
Encephahtozoon cuniculi
Nosema helminthorum
Unikaryon legeri

The bacterium *Salmonella typhimurium*, the bacterium *Mycobacterium avium*, and the protozoan *Leishmania amazonensis* are each particularly useful vectors for the present invention, since each of these organisms shows natural preference for attachment to and penetration into certain solid tumor cancer cells in tissue culture, as opposed to non-cancerous counterpart cells. Since these vectors, such as *Salmonella*, have a natural ability to distinguish between cancerous cells and their non-cancerous counterpart cells they are directly applicable to the methods for diagnosis or treatment according to the present invention. However, this tumor-specific ability, as well as, the ability to be super-infective as compared to the "wild type" parent strain may be enhanced and selected for by using the methods of the present invention described in Sections 6.1.1-6.1.4., *infra*.

6.1.1. ISOLATION BY CYCLING THROUGH *IN VITRO* TISSUE CULTURE

One embodiment of the present invention is to isolate the novel vectors of the present invention by cycling a microorganism through a pre-selected target cell, preferably a solid tumor cancer cell, with one or more cycles of infection in *in vitro* tissue culture so that the cycled population and/or clonal isolates therefrom demonstrate enhanced infectivity of the target tumor cell as compared to the starting microbial population and enhanced selectivity as compared to the non-cancerous counterpart cell. The method entails selecting a parasite or pathogen and adding the microorganism to an *in vitro* tissue culture system of the particular type of solid tumor that one wishes to use as a target cell. For example, if one desires to target melanoma tumors, the target cell may be M2 human melanoma cells. After incubating the tumor cells and microorganisms together, which allows enough time for the microorganism to attach and/or infect the tumor cell, the tumor cell culture is washed with either buffer or medium which contains an antibiotic agent effective against the specific microorganism

used. The antibiotic agent kills any microorganisms that have not attached to and infected the tumor cell. If desired, the infected tumor cell culture may be incubated further in medium containing antibiotic for varying times, depending on the type of population of microorganisms to be isolated. For example, for longer incubation times, the microorganism population isolated has enhanced survival and/or proliferative abilities inside the tumor cells as compared to the starting population of microorganisms. Additionally, the isolated populations can be cultured to isolate single colony clones using standard techniques.

The infected animal cells are collected and lysed, thus freeing the internalized microorganisms. The microorganism can then be isolated, for example, by centrifugation (2000x g for 4 minutes) and resuspending in fresh medium. The isolated microbial population may then be used for additional cycles of infection into and isolation out of the target tumor cell. The isolated microbial population may be placed first in appropriate growth medium for 1-2 doubling times before being subjected to additional infection cycles to insure their viability. The isolated microbial population may also be cultured so as to isolate and collect single colony clones. The isolated microorganisms may also undergo known *in vitro* techniques to determine their relative infective and selective abilities as compared to the "wild type" parent strain which did not undergo *in vitro* selection. For example, in side by side comparisons one may test the relative infectiveness of the isolated microorganism as compared to the "wild type" by using assays designed to quantitate the number of microorganisms which have attached to or invaded the target tumor cell and/or their ability to distinguish between cancerous and non-cancerous cells. In addition, parameters such as microorganism population density, may be varied in these *in vitro* assays which assists in determining what effect the overall concentration of inoculum of the clone or population being tested has on the ability of the

microorganisms to differentiate between the target tumor cells and their non-cancerous counterparts.

For an illustrative example of super-infective, tumor-specific vectors isolated by cycling through *in vitro* tissue culture, see Section 7, *infra*.

6.1.2. ISOLATION BY CYCLING THROUGH *IN VIVO* SOLID TUMORS

Another embodiment of the present invention is to isolate the novel vectors of the present invention by cycling the microorganism through solid tumors *in vivo*. This procedure is performed using experimental tumor models in mammals such as, for example and not by way of limitation, B16 mouse melanoma cells which form melanoma tumors in C57B6 mice and HCT116 human colon carcinoma cell which form colon carcinomas in *nu/nu* and other immuno-compromised mice. Additionally, fresh biopsies of tumor tissue which are obtained surgically from a cancer patient may be used to inoculate *nu/nu*, *scid* or other immuno-compromised mice. These tumors in mice which have grown from inoculated cancer cells are used as *in vivo* targets for the isolation of super-infective and tumor-specific vectors in a similar manner as *in vitro* target cells. Any tumor growing in mice or any other animal may be used in the present invention as a target for the isolation of super-infective and tumor-specific vectors *in vivo*.

Once the tumor is established in the mouse, by, for example, inoculation of cancer cells sub-cutaneously or transplantation of a tumor mass, the selected microorganism is inoculated into the mouse. After a pre-determined infection time after inoculation in which the microorganism becomes co-localized with the tumor and/or infects the tumor cells, the mice are sacrificed, the tumors excised, weighed and homogenized. An aliquot may be diluted into the proper microorganism growth medium and incubated at the proper growth conditions for 1-2 population doublings to insure the recovery of viable microorganisms for successive inoculations

into tumor bearing mice. Further, if the isolated population is to undergo successive inoculations in tumor bearing mice, upon each successive inoculation, the number of microorganisms in the inoculate and the time of infection may be reduced to increase the stringency of selection for tumor-specific isolates. Additionally, the isolated populations can be cultured to isolate single colony clones using standard techniques. The isolated microorganisms may be used also in *in vitro* assays to determine their relative infective and selective abilities as compared to the "wild type" parent strain which did not undergo an *in vivo* selection procedure.

For an illustrative example of super-infective, tumor-specific vectors isolated *in vivo* in tumor-bearing mice, see Section 9, *infra*.

6.1.3. ISOLATION BY *IN VITRO* CHEMOTAXIS USING MEDIUM CONDITIONED BY THE TARGET TUMOR CELL

Another embodiment of the present invention is to provide methods for isolating super-infective and/or tumor cell-specific vectors by chemotaxis so that the isolated microorganisms have increased chemotactic ability towards tumor cell secretory products. The method entails using capillary tubes which are loaded with either liquid control medium or medium that has been conditioned by the target tumor cell as described by Adler (1973, J. General Microbiology 74:77-91). Conditioned medium is medium in which the target cells have been grown and subsequently has been filtered to remove the cells. One end of the capillary is sealed in a flame; the capillary is then quickly passed several times through a flame and is immediately plunged open end down into a beaker containing either the conditioned or control medium. As the capillaries cool, the liquid is drawn up inside.

The loaded capillary tubes are inserted open end down into a centrifuge tube containing medium and a suspension of the pre-selected microorganism. After a pre-

determined period of incubation at 37°C in which the microorganism chemotacts into the capillary tubes, the capillary tubes are removed with forceps, the sealed ends are opened and the opened capillaries are transferred into centrifuge tubes containing nutrient medium appropriate for the particular microorganism. It is important that the upper tips of the capillary tubes are covered with an appropriate medium for the particular type of microorganism to assure quantitative recovery of the microorganism from the capillary tubes during centrifugation. The capillary tubes are centrifuged, for example, at 4000x g for 4 minutes, to force the microorganism out of the tube. The capillary tubes are removed, the microorganism resuspended, and an aliquot spread onto the appropriate medium in either solid or liquid form to allow for quantitation.

Significant increases in the number of microorganisms entering into the capillary tubes containing conditioned medium as compared to controls indicates a positive chemotactic response toward secreted products of the target cell found in the conditioned medium. The populations isolated by this in vitro technique can undergo successive chemotaxis assay isolation or be used to isolate single colony clones. These clones or populations can be compared to the "wild type" parent strain in their ability to distinguish between the target cancerous cell and the non-cancerous counterpart cell as well as for super-infective ability.

For an illustrative example of super-infective, tumor-specific vectors isolated by *in vitro* chemotaxis using tumor cell-conditioned medium, see Section 8, *infra*.

6.1.4. ISOLATION OF MUTAGENIZED VECTORS

In any of the above-described methods for isolating super-infective, tumor-specific microorganisms, the "wild type" parent microorganism can be subjected first to mutagenesis before the microorganism is subjected to any isolation or selection procedure of the present invention.

For example, bacteria are subjected to treatment with nitrosoguanidine and ultraviolet B irradiation so that the hereditary genetic material is modified resulting in the altered expression of genes, both qualitatively and quantitatively, in the microorganism. Other types of chemical and high-energy mutagenesis are well known in the art. For example, alkylating agents such as dimethyl nitrosamine or ethyl methane sulfonate, or intercalating agents, such as ethidium bromide. Other approaches include transposon mutagenesis to introduce genetic flocks or fusions of genes with new promoters. Any mutagen can be used in the present invention to create mutant strains of microorganisms which may then undergo any of the selection methods of the present invention.

For an illustrative example of mutagenesis, see Section 7.1, *infra*.

6.2. GENETIC MANIPULATION OF THE SELECTED VECTORS FOR DELIVERY OF GENES AND/OR GENE PRODUCTS TO THE TARGET SOLID TUMOR CELLS AS WELL AS FOR ATTENUATION OF VIRULENCE

6.2.1. GENETIC MANIPULATION FOR DELIVERY OF GENES AND/OR GENE PRODUCTS TO THE TARGET SITE

After the selection processes described above in which one obtains a super-infective, tumor-specific vector, one can genetically engineer such vectors so that any desired gene or gene product is delivered to a target site, preferably the site of a solid tumor, more preferably, into the tumor cell itself, the necrotic areas of the tumor or into tumor-associated lymphocytes and macrophages. Additionally, one can genetically alter naturally occurring microorganisms which have a natural ability to infect tumor cells and/or be tumor-cell specific. These vectors are genetically engineered by a wide variety of methods known in the art, for example, transformation or electroporation. In a preferred embodiment of the present invention the vectors

are engineered to deliver suicide genes to the target tumor cells. These suicide genes include pro-drug converting enzymes, such as *Herpes simplex* thymidine kinase (TK) and bacterial cytosine deaminase (CD). TK phosphorylates the non-toxic substrates acyclovir and ganciclovir, rendering them toxic via their incorporation into genomic DNA. CD converts the non-toxic 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), which is toxic via its incorporation into RNA. Additional examples of pro-drug converting enzymes encompassed by the present invention include cytochrome p450 NADPH oxidoreductase which acts upon mitomycin C and porfiromycin (Murray et al., 1994, J. Pharmacol. Exp. Therapeut. 270:645-649).

Prodrug converting enzymes are being widely employed for use in gene therapy of malignant cancers (Vile and Hart, 1993, Cancer Res. 53:3860-3864; Moolten and Wells, 1990, J. Natl. Cancer Inst. 82:297-300; Wagner, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1441-1445; Mullen, 1994, Cancer Res. 54:1503-1506; Huber et al., 1993, Cancer Res. 53:4619-4625; Waldman et al., 1983, J. Biol. Chem. 258:11571-11575; Mullen, et al., 1992, Proc. Natl. Acad. Sci. 89:33-37; Austin and Huber, 1993, Mol. Pharmacol. 43:380-387). Table 2 is an illustrative list of pro-drug converting enzymes (Bagshawe, 1995, Drug Dev. Res. 34:220-230).

Prodrug converting enzymes have been expressed in several bacteria. The *Herpes simplex* virus has been expressed in *E. coli* (Garapin, 1980, Proc. Natl. Acad. Sci. USA 78:815-819; Waldman et al., 1983, J. Biol. Chem. 258:11571-11575). Similarly, Simula et al., 1993, Toxicology 82:3-20, expressed the prodrug converting enzyme cytochrome p450 oxidoreductase in *Salmonella typhimurium* which confered sensitivity to mitomycin.

TABLE 2

REPRESENTATIVE PRO-DRUG CONVERTING ENZYMES FOR USE IN VECTOR THERAPY

Enzyme	Pro-drug	Reference
Carboxypeptidase G2	benzoic acid mustards	Bashawe et al., 1988; Springer et al., 1990
	aniline mustards	Davies et al., 1994
	phenol mustards	Springer et al.
Beta-glucuronidase	p-hydroxyaniline mustard-glucuronide	Roffer et al., 1991
	epirubicin-glucuronide	Halsma et al., 1992
		Mitaku et al., 1994
Penicillin-V-amidase	adriamycin-N phenoxyaceryl	Kerr et al., 1990
Penicillin-G-amidase	N-(4'-hydroxyphenyl acetyl)-palytoxin doxorubicin	Bignami et al., 1992
	melphalan	Vrudhula et al., 1993
β-lactamase	nitrogen mustard-cephalosporin	Alexander et al., 1991
	β-phenylenediamine	
	vinblastine derivative-cephalosporin	
	cephalosporin mustard	Meyer et al., 1993
		Svensson et al, 1993
β-glucosidase	cyanophenylmethyl-β-D-gluco-pyranosiduronic acid	Rowlandson-Busza et al., 1991
Nitroreductase	5-(adaridin-1-yl-)2, 4-dinitrobenzamide	Knox et al., 1988; Somani and Wilman, 1994
Carboxypeptidase A	methotrexate-alanine	Haenseler et al., 1992

Bagshawe et al., 1988, Br. J. Cancer 58:700-703.

Springer et al., 1990, J. Med. Chem. 33:677-681.

Davies et al., 1994, Ann. Oncol. 5 (Suppl 5):73(abstr).

Springer et al., 1994, A novel bisiodo-phenol mustard in antibody-directed enzyme pro-drug therapy (ADEPT). In: Programme of Eleventh Hammersmith Conference. Advances in the Application of Monoclonal Antibodies. London: Hammersmith Hospital (abstr).

Haisma et al., 1992(a), Cancer Immunol. Immunother. 34:343-348.

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Haenseler, E., Esswein, A., Vitols, K.S., Montejano, V., Mueller et al., 1992, Biochemistry 31:214-220.

However, pro-drug converting enzymes such as the TK and CD enzymes, when synthesized in bacteria such as *Salmonella* or *Escherichia coli*, are not normally secreted from the bacteria. Accordingly, the expression construct is designed such that the microorganism-produced gene products are secreted by the microorganism. Thus, TK or CD are able to generate phosphorylated acyclovir, ganciclovir, or 5-FU, within the target tumor cell cytoplasm and interstitial spaces of the target tumor. Secretion of TK and CD is achieved by introducing into the expression construct a secretory signal sequence, for example, from the β-lactamase gene (Talmadge et al., 1980, Proc. Natl. Acad. Sci. USA 77:3369-3373).

Alternate signal sequences, in addition to β-lactamase, are also encompassed by the present invention.

Bacteria, for example, are known to have several means for secretion into the periplasm and the outside media. The most typical secretion sequences are N-terminal signal sequences containing hydrophobic transmembrane spanning domains. These sequences serve to guide the protein through the membrane and are removed as- or after the protein crosses the membrane. Prokaryotic and eukaryotic N-terminal signal sequences are similar and it has been shown that eukaryotic N-terminal signal sequences are capable of functioning as secretion sequences in bacteria. In a preferred embodiment, the gene encoding the enzyme β-lactamase (penicillinase) is used as the source of the signal sequence. This signal sequence is a well studied example of a bacterial enzyme which is secreted both into the periplasm and into the external media.

Further, some bacterial proteins utilize a different secretion signal which is located at the C-terminus. The enteroinvasive *E. coli* hemolysin A *(hlyA)* is the best studied member of this group. It has been shown that the secretion signal is present in the last 60 amino acids of that protein and that transfer of this domain to other proteins can result in their direct secretion into the media when the accessory proteins from the hemolysin operon (*hylC, A, B, & D*) are present (Su et al., 1992, Microbial Pathogen. 13:465-476). An illustrative list of secreted proteins reviewed by Pugsley is presented in Table 3, (Pugsley A. P., 1988, Protein secretion across the outer membrane of gram-negative bacteria. In: Protein Transfer and Organelle Biogenesis, R.C. Dand and P. W. Robbins (eds), Academic Press, Inc., Harcourt Brace Jovanovich, Publishers, San Diego, pp 607-652).

TABLE 3

SOURCES OF SECRETION SIGNALS FOR PRO-CONVERTING ENZYMES

Protein	Organism	Location in Transfected *E. coli* and type of signal	Ref. No.
Chitinase	*Serratia marcescens*	released into medium N-terminal signal	14
α-Hemolysin	*E. coli*	released into medium C-terminal signal	9
Heat labile enterotoxin I	various *E. coli* strains	Similar to cholera toxin 2 subunits (A&B); N-terminal signal in both; primarily in periplasm	12, 7, 29
Heat-stable enterotoxin I	various *E. coli* strains	N-terminal signal peptide; secreted into the media	11, 28
Heat-labile enterotoxin II	various *E. coli* strains	N-terminal signal peptide	17
Pullulanase	*Kelbsiella pneumoniae*	Release into the medium; N-terminal signal peptide	3, 27, 6
Serine protease	*S. marcencens*	Secreted into the medium; N-terminal signal peptide	31
Pectate lyase	*Erwinia chrysanthemi*	Mainly in the periplasm	15, 5
Pectate lyase	*E. carotovara*	Periplasm	18, 32
Protease	*E. chrysanthemi*	Secreted into the medium	30, 1
Aerolysin	*Aeromonas hydrophila*	Periplasm, N-terminal signal sequence (processed)	13
Phospholipase C	*Pseudomonas aeruginosa*	not secreted by *E. coli* N-terminal signal sequence	4, 19, 26
Exotoxin A	*P. aeruginosa*	not secreted by *E. coli*	10
Cholera toxin	*Vibrio cholerae*	Mainly periplasmic; 2 subunits	25, 20
Hemolysin	*V. cholerae*	Periplasm	21
DNase	*V. Cholerae*	Periplasm	22, 8
Thermostable Hemolysin	*V. parahaemolyticus*	Periplasm N-terminal signal peptide	24

TABLE 3

IgA protease	*Haemolphilis influenzae*	Periplasm	2
IgA protease	*Nisseria gonorrhoeae*	Secreted into the medium	16
Pertussis toxin	*Bordetella pertussis*	Periplasm; 5 subunits all with N-terminal signal peptides	23

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2. Bricker et al., 1983, Proc. Natl. Acad. Sci. USA 80:2681-2685.
3. Chapon et al., 1985, J. Bacteriol. 164:639-645.
4. Coleman et al., 1983, J. Bacteriol. 153:909-915.
5. Collmer et al., 1985, J. Bacteriol. 161:913-920.
6. d'Enfert et al., 1987, EMBO J. 6:3531-3538.
7. DaRas et al., 1980, Nature 288:499501.
8. Focareta et al., 1985, FEMS Mcrobiol. Lett. 29:161-166.
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10. Gray et al., 1984, Proc. Natl. Acad. Sci. USA 81:2645-2649.
11. Guzmin-Verduzco et al., 1983, J. Bacteriol. 154:146-151.
12. Hirst et al., 1984, Proc. Natl. Acad. Sci. USA 81:7752-7756.
13. Howard et al., 1986, Mol. Gen. Genet. 204:289-295.
14. Jones et al., 1986, EMBO J. 5:2377-2383.
15. Keen et al., 1984, J. Bacteriol. 159:825-831.
16. Koomey et al., 1982, Proc. Natl. Acad. Sci. USA 79:7881-7885.
17. Lee et al., 1983, Infect. Immun. 42:264-268.
18. Lei et al., 1985, Gene 35:63-70.
19. Lory et al., 1983, Gene 22:95-101.
20. Mekalanos et al., 1983, Nature 306:551-557.
21. Mercuric et al., 1985, Mol. Gen. Genet. 200:472475.
22. Newland et al., 1985, Infect. Immun. 47:691-696.
23. Nicosia et al., 1986, Proc. Natl. Acad. Sci. USA 83:4631-4635.
24. Nishibuchi et al., 1985, J. Bacteriol. 162:558-564.
25. Pearson et al., 1982, Proc. Natl. Acad. Sci. USA 79:2976-2980.

26. Pritchard et al., 1986, J. Bacteriol. 167:291-298.

27. Pugsley, A. P., 1988, Protein secretion across the outer membrane of gram-negative bacteria. In: Protein Transfer and Organelle Biogenesis, R.C. Dand and P. W. Robbins (eds), Academic Press, Inc., Harcourt Brace Jovanovich, Publishers, San Diego, pp 607-652.

28. So et al., 1980, Proc. Natl. Acad. Sci. USA 77:4011-4015.

29. Spicer et al., 1982, J. Biol. Chem. 257:5716-5721.

30. Wandersman C. (unpublished results cited in Pugsley, 1988).

31. Yanigida et al., 1986, J. Bacteriol. 166:937-944.

32. Zink et al., 1985, Appl. Environ. Microbiol. 49:714-717.

In another embodiment of the present invention, the desired genes expressed from the expression constructs are under the specific regulatory control of certain types of promoters. These promoters may be either constitutive, in which the genes are continually expressed, inducible, in which the genes are expressed only upon the presence of an inducer molecule(s) or cell-type specific control, in which the genes, including but not limited to suicide genes, are expressed only in certain cell types. Further, expression of foreign genes including prodrug converting enzymes frequently alters the phenotype of the bacteria. Therefore, it would be an advantage to drive the expression of a prodrug converting enzyme under exogenous control. This would allow exploitation of the bacterial phenotypes such as tumor targeting and amplification, after which time it would be beneficial to express the prodrug enzyme. Inducible promoters drive gene expression under specific conditions. Furthermore, exogenously inducible promoters respond to specific stimuli including chemical signals which can be artificially introduced. It would be an advantage to drive the expression of a prodrug enzyme using an exogenously introduced agent which is approved for use in humans. The "SOS" response of bacteria (Friedberg et al., In: DNA Repair and Mutagenesis, pp. 407-455, Am. Soc. Microbiol. Press, 1995) is a response inducible by numerous agents including chemotherapeutic alkylating agents such as mitomycin (Oda et

al., 1985, Mutation Research 147:219-229; Nakamura et al., 1987, Mutation Res. 192:239-246; Shimda et al., 1994, Carcinogenesis 15:2523-2529) which is approved for use in humans. Promoter elements which belong to this group include *umuC*, *sulA* and others (Shinagawa et al., 1983, Gene 23:167-174; Schnarr et al., 1991, Biochemie 73:423-431). The *sulA* promoter includes the ATG of the *sulA* gene and the following 27 nucleotides as well as 70 nucleotides upstream of the ATG (Cole, 1983, Mol. Gen. Genet. 189:400-404). Therefore, it is useful both in expressing foreign genes and in creating gene fusions for sequences lacking initiating codons.

In one embodiment, for example, the expression of the gene is controlled by a bacterial promoter which is activated in specific target cells. In a preferred mode of this embodiment, the bacterial promoter is activated primarily in specific target cells. In another embodiment, for example, the expression of the gene is controlled by a bacterial promoter which is activated only in specific tumor cells. An illustrative example of an expression construct which expresses a gene under the control of a promoter with the necessary secretion signal sequence is diagrammed in Figure 1.

In a preferred embodiment of the present invention, the expression of the gene is under the control of a promoter which is active only in the target cell. Microorganism promoters that are specifically or preferentially active in a target tumor cell are isolated by a number of different methods. For example, one method is using IVET (*in vivo* expression technology) promoter trap procedure for isolating specifically induced genes. This procedure is carried out by taking, for example, a random pool of *Salmonella typhimurium* DNA insertions generated by *Sau3A* restriction enzyme and cloning the fragments into the promoter trap vector pIVET (Slauch et al., 1994, Methods Enzymol. 235:481-492; Mahan et al., 1993, Science 259:686-688). The cloning site is at the position of the promoter for the *purA* gene which is required for the synthesis of cyclic AMP. This representative pool is

transfected back into *Salmonella typhimurium* and an integration event is induced which results in replacement of the endogenous *purA* gene. The population of bacteria carrying an integrated IVET plasmid is allowed to infect an animal bearing a solid tumor of the cell type of choice and after 24 hours bacteria are isolated from the tumor. Only those bacteria that received a plasmid whose random piece of *Sau3A* restricted DNA acts as a promoter within the tumor cells is capable of surviving. In addition to controlling the transcription of *purA*, the *Sau3A* restricted DNA promoter also controls the expression of the β-galactosidase gene. Two types of promoters are isolated which allow the survival of bacteria within tumors, constitutive and regulated. The constitutive promoter continues to control the positive expression of both genes, inside and outside of the tumor. Whereas the regulated promoter is no longer active in cells other than the target cell.

Another method for identifying promoters that are active in tumors is to identify tumor-specifically induced microbiological gene products using two dimensional gel electrophoresis. For example, to determine which gene products are specifically or preferentially expressed in melanoma cells rather than macrophages, the method entails three parallel infections which proceed in tandem: (1) 5×10^7 clonal microorganisms are allowed to infect 5×10^6 melanoma cells, (2) 5×10^7 clonal microorganisms are allowed to infect 5×10^6 macrophages, and (3) 5×10^7 clonal microorganisms are maintained in growth phase in LB broth. After a 30 minute infection the cells are washed with DMEM with 10 μg/ml gentamicin (for melanoma cells), RPMI 1640 with 10 μg/ml gentamicin (for macrophages) and LB without gentamicin (for free microorganisms). After two hours the cells are pretreated with 50 mg/ml cyclohexamide to inhibit host cell protein synthesis for 15 minutes. The cells are then washed and placed in labeling medium (minus methionine) containing 75 μCi/ml ^{35}S-methionine for 30 minutes, followed by 1 hour in normal medium. The cells are then harvested, denatured in 7M

urea buffer and subjected to isoelectrofocusing (IEF) followed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and analysis by autoradiography. Gene products specifically expressed in melanoma cells appear as protein spots from microorganism-infected melanomas but not from microorganism-infected macrophages or from free microorganisms. The microorganismal genes that are specifically expressed are cloned from a λgt11 expression library using antiserum prepared from proteins derived from preparative IEF and SDS-PAGE gels. Subsequent cloning from a cosmid library results in DNA fragments containing the promoter elements for the tumor-specific expressed gene product.

Yet another method for isolating promoters which are specifically or preferentially activated in the target tumor cells is transposon mutagenesis. Transposon mutagenesis results in a pool of random mutants which can be tested for their ability to survive in epithelial cells but not in target tumor cells. Mutants are first tested for their continued ability to persist in epithelial cells. Mutants no longer able to survive will be selected against. Surviving mutants are picked at random and placed in a numbered array using 96 well plates. The target tumor cells are grown in 96 well plates and individually infected with a microorganism at a microorganism to host ratio of about 10:1 for 30 minutes, followed by washing and treatment with 10 μg/ml gentamicin. After 24 hours the plates are rinsed and stained with 0.4% trypan blue to determine the ratio of living cells(clear) to dead cells (blue) using a 96 well plate reader. Microorganisms which are unable to survive within the target tumor cell are recovered from the original numbered plate. The genes are then cloned using the transposon as a genetic marker to isolate the DNA containing the tumor-specific expressed gene and its promoter.

The vectors which can express the various pro-drug or "suicide genes" when given to the host should not confer antibiotic resistance to the host and more importantly the

bacteria should remain as sensitive to as many antibiotics as possible. Therefore, these vectors should not carry any antibiotic resistance markers. This can pose a problem in maintaining the expression vectors in the bacteria in absence of selective pressure. However, the are a number of methods in which the vectors can be stably maintained without resorting to antibiotic resistance. For example, one such method is the construction of chromosomally integrated vectors expressing pro-drug converting enzymes or other "suicide genes" as described by Donnenberg, 1991, Am. Soc. Microbiol., Annual Meeting, Abstract B-111, p.4; Donnenberg and Kaper, 1991, Infect. Immun. 59:4310-4317; and Ried and Collmer, 1987, Gene 57:239-246. Another method is the construction of stable episomal plasmids encoding "suicide genes" or pro-drug converting enzymes using a balanced lethal system. Such balanced lethal systems are defined by the fact that the vector encodes for a function that compensates for a deficiency in the bacteria, such that the presence of the vector is essential for the survival of the bacteria. Such a system is described by Galan et al., 1990, Gene 94:29-35. This system has the advantage over chromosomal integration in that the plasmids are multicopy and, therefore, achieve higher expression levels.

6.2.2. GENETIC MANIPULATION FOR ATTENUATION OF VIRULENCE

Many of the microorganisms encompassed by the present invention are causative agents of diseases in humans and animals. For example, sepsis from gram negative bacteria is a serious problem because of the high mortality rate associated with the onset of septic shock (R.C. Bone, 1993, Clinical Microbiol. Revs. 6:57-68). Therefore, to allow the safe use of these vectors in both diagnostics and treatment of humans and animals, the microorganism vectors are attenuated in their virulence for causing disease. In the present invention, attenuation, in addition to its traditional definition in which a microorganism or vector is

modified so that the microorganism or vector is less pathogenic, is intended to include also the modification of a microorganism or vector so that a lower titer of that derived microorganism or vector can be administered to a patient and still achieve comparable results as if one had administered a higher titer of the parental microorganism or vector. The end result is to reduce the risk of toxic shock or other side effects due to administration of the vector to the patient. Such attenuated microorganisms are isolated in a number of techniques. Such methods include use of antibiotic-sensitive strains of microorganisms, mutagenesis of the microorganisms, selection for tumor-specific, super-infective microorganism mutants in culture or in tumor-bearing animals, selection for microorganism mutants that lack virulence factors necessary for survival in normal cells, including macrophages and neutrophils, and construction of new strains of microorganisms with altered cell wall lipopolysaccharides. For example, in Section 6.1 *et seq.* where methods are described for the isolation of super-infective, tumor-specific vectors, these same methods are also methods for isolating attenuated vectors; super-infective, tumor cell-specific vectors are by definition attenuated. As the vectors are highly specific and super-infective, the difference between the number of infecting bacteria found at the target tumor cell as compared to the non-cancerous counterparts becomes larger and larger as the dilution of the microorganism culture is increased such that lower titers of microorganism vectors can be used with positive results.

Further, the microorganisms can be attenuated by the deletion or disruption of DNA sequences which encode for virulence factors which insure survival of the microorganisms in the host cell, especially macrophages and neutrophils, by, for example, homologous recombination techniques and chemical or transposon mutagenesis. For example, a number of these virulence factors have been identified in *Salmonella*. Many, but not all, of these studied virulence factors are associated with survival in macrophages such that these

factors are specifically expressed within macrophages due to stress, for example, acidification, or are used to induced specific host cell responses, for example, macropinocytosis, Fields et al., 1986, Proc. Natl. Acad. Sci. USA 83:5189-5193. Table 4 is an illustrative list of *Salmonella* virulence factors which, if deleted by homologous recombination techniques or chemical or transposon mutagenesis, result in attenuated *Salmonella*.

TABLE 4

REPRESENTATIVE VIRULENCE FACTORS FOR *SALMONELLA TYPHIMURIUM* AND OTHER BACTERIA

Virulence Factor or Loci, Specific Stress Overcome or Stimulated Response	Reference
Acidification	Alpuche-Aranda et al., 1992
5'-adenosine monophosphate	Biochenko and Levashev, 1987
Cytolysin	Libbey et al., 1994
Defensin resistance loci	Fields et al., 1989
DNAK	Buchmeier and Hefferon, 1990
Fimbriae	Ernst et al., 1990
GroEL	Buchmeier and Hefferon, 1990
Induced Macropinocytosis	Alpuche Aranda, et al., 1994 Ginocchio et al., 1992 Jones et al., 1993
Inv loci	Betts and Finlay, 1992 Galon and Curtis III Ginocchio et al., 1992
Lipoprotein	Stone et al., 1992
LPS	Gianeiella et al., 1973 Stone et al., 1992
Lysosomal fusion inhibition	Ishibashi et al., 1992
Macropage survival loci	Fields et al., 1989
Oxidative stress (Sox; in *E. coli*)	Nunoshiba et al., 1993
PhoP and PhoQ	Behlau and Miller, 1993 Groisman et al., 1993 Miller et al., 1989

TABLE 4

Pho activated genes (pag; *e.g.*, pagB and pagC)	Abshire and Neidhardt, 1993 Hefferon et al., 1992 Miller et al., 1992 Miller et al., 1989 Pulkkinen and Miller, 1991 Stone et al., 1992
PhoP and PhoQ regulated genes (*prg*)	Miller et al., 1989 Behlau and Miller, 1993; 1994
Porins	Tufano et al., 1988
Serum resistance peptide	Hackett et al., 1987
Virulence factors	Abshir and Neidhardt, 1993 Loos and Wassenaar, 1994 Mahan et al., 1995 Sansonetti, 1992
Virulence plasmid	Gulig and Curtiss, 1987 Rhen et al., 1993 Riikonen et al., 1992
spvB (virulence plasmid) traT (virulence plasmid) ty2	Fierer et al., 1993 Rhen and Sukupolvi, 1988 Elsinghorst et al., 1989

Abshiro et al., 1993, J. Bacteriol. 175:3734-3743

Alpuche-Aranda et al., 1992, Proc. Natl. Acad. Sci. USA 89:10079-83

Alpuche-Aranda et al., 1994, J. Exp. Med. 179:601-6088

Baumler et al., 1994, Infect. Immun. 62:1623-1630

Behlau et al., 1993, J. Bacteriol. 175:4475-4484

Belden et al., 1994, Infect. Immun. 62:5095-5101

Betts et al., 1992, Can. J. Microbiol. 38:852-7

Boichenko et al., 1987, Bull. Eksp. Biol. Med. 103:190-2

Boichenko et al., 1988, Zh. Mikrobiol. Epidemiol. Immunobiol. 7:9-11

Boichenko et al., 1985, Zh. Mkrobiol. Epidemiol. Immunobiol. 12:67-9

Bowe et al., 1994, Methods Enzymol. 236:509-26

Buchmeier et al., 1989, Infect. Immun. 57:1-7

Buchmeier et al., 1990, Science 248:730-732

Buchmeier et al., 1995, J. Cldn. Invest. 95:1047-53

Buchmeier et al., 1993, Mol. Microbiol. 7:933-936

Dragunsky et al., 1989, J. Biol. Stand. 17:353-60

Emoto et al., 1993, J. Immunol. 150:3411-3420

Ernst et al., 1990, Infect. Immun. 58:2014-2016

Elsinghorst et al., 1989, Proc. Natl. Acad. Sci. USA 86:5173-5177

Fields et al., 1986, Proc. Natl. Acad. Sci. USA 83:5189-93

Fields et al.,1989, Science 243:1059-62

Fierer et al., 1993, Infect. Immun. 61:5231-5236

Gianella et al., 1973, J. Infect. Dis. 128:69-75

Galan et al., 1989, Microb. Pathog. 6:433-443

Galan et al., 1990, Infect. Immun. 58:1879-1885

Ginocchio et al., 1992, Proc. Natl. Acad. Sci. USA 89:5976-5980

Gulig et al., 1987, Infect. Immun. 55:2891-901

Hackett et al., 1987, J. Infect. Dis. 155:540-549

Heffernan et al., 1992, J. Bacteriol. 174:84-91

Ishibashi et al., 1992, Microb. Pathog. 13:317-323

Libby et al., 1994, Proc. Natl. Acad. Sci. USA 91:489-493

Loos et al., 1994, Immun. Infekt. 22:14-19

Mahan et al., 1995, Proc. Natl. Acad. Sci. USA 92:669-673

Miller et al., 1989, Proc. Natl. Acad. Sci. USA 86:5054-5058

Miller et al., 1992, Infect. Immun. 60:3763-3770

Nunoshiba et al., 1993, Proc. Natl. Acad. Sci. USA 90:9993-9997

Pollack et al., 1986, Nature 322:834-836

Pulkkinen and Miller, 1991, J. Bacteriol. 173:86-93

Rhen et al., 1993, Mol. Microbiol. 10:45-56

Rhen et al., 1988, Microb. Pathog. 5:275-285

Riikonen et al., 1992, Microb. Pathog. 13:281-291

Sansonetti, 1992, Rev. Prat. 42:2263-2267

Stone et al., 1992, J. Bacteriol. 174:3945-3952

Tufano et al., 1988, Eur. J. Epidemiol. 4:110-114

Yet another method for the attenuation of the isolated vectors is to modify substituents of the microorganism which are responsible for the toxicity of that microorganism. For example, lipopolysaccharide (LPS) or endotoxin is primarily responsible for the pathological effects of bacterial sepsis. The component of LPS which results in this response is lipid A (LA). Elimination or mitigation of the toxic effects of LA results in an attenuated bacteria since 1) the risk of septic shock in the patient would be reduced and 2) higher levels of the bacterial vector could be tolerated. *Rhodobacter (Rhodopseudomonas) sphaeroides* and *Rhodobacter capsulatus* each possess a monophosphoryl lipid A (MLA) which does not elicit a septic shock response in experimental animals and, further, is an endotoxin antagonist. Loppnow et al., 1990, Infect. Immun. 58:3743-3750; Takayma et al., 1989, Infect. Immun. 57:1336-1338.

Known similarities in lipid metabolism and genetic organization of lipid metabolic genes between *Rhodobacter sphaeroides* and other gram negative bacteria and the ability of Rhodobacter genes to complement *E. coli* mutations (Benning and Somerville, 1992(A), J. Bacteriol. 174:6479-6487; 1992(B), J. Bacteriol. 174:2352-2360; Carty et al., 1994, FEMS Microbiol. Lett. 118(3):227-231) demonstrate that, for

example, *Salmonella* and other bacteria can be genetically altered to produce MLA, thereby reducing its potential of inducing septic shock. A preferred embodiment of the present invention is a *Salmonella* spp. strain that expresses MLA rather than LA and also expresses *HSV* TK under the control of a tumor-specific promoter.

As an illustrative example, the generation of MLA producing *Escherichia coli* or *Salmonella typhimurium* entails constructing a DNA gene library composed of 10 kB fragments from *Rhodobacter sphaeroides* which is generated in λgt11 or pUC19 plasmids and transfected into *E. coli*. Clones which produce MLA are positively selected by using an antibody screening methodology to detect MLA, such as ELISA. In another example one generates a cosmid library composed of 40 kB DNA fragments from *Rhodobacter sphaeroides* in pSuperCos which is then transfected into *Salmonella typhimurium*. Clones which produce MLA are positively selected by using an antibody screening methodology to detect MLA, such as ELISA.

Yet another example for altering the LPS of *Salmonella* involves the introduction of mutations in the LPS biosynthetic pathway. Several enzymatic steps in LPS biosynthesis and the genetic loci controlling them in both *E.coli* and *Salmonella typhimurium* have been identified (Raetz, 1993, J. Bacteriol. 175:5745-5753 and references therein). Several mutant strains of *Salmonella typhimurium* and *E. coli* have been isolated with genetic and enzymatic lesions in the LPS pathway. One such mutant, *firA* is a mutation within the gene that encodes the enzyme UDP-3-O(R-30 hydroxymyristoyl)-glycocyamine N-acyltransferase, that regulates the third step in endotoxin biosynthesis (Kelley et al., 1993, J. Biol. Chem. 268:19866-19874). *Salmonella typhimurium* and *E.coli* strains bearing this type of mutation produce a lipid A that differs from wild type lipid A in that it contains a seventh fatty acid, a hexadecanoic acid (Roy and Coleman, 1994, J. Bacteriol. 176:1639-1646). Roy and Coleman demonstrated that in addition to blocking the third step in endotoxin biosynthesis, the *firA*$^-$ mutation also

decreases enzymatic activity of lipid A 4' kinase that regulates the sixth step of lipid A biosynthesis.

Once the strain has been attenuated by any of the methods known in the art, the stability of the attenuated phenotype is important such that the strains do not revert to a more virulent phenotype during the course of treatment of a patient. Such stability can be obtained, for example, by providing that the virulence gene is disrupted by deletion or other non-reverting mutations on the chromosomal level rather than epistatically or that the "suicide gene" is stably integrated into the bacterial chromosome.

Another method of insuring the attenuated phenotype is to engineer the bacteria such that it is attenuated in more than one manner, *e.g.*, a mutation in the pathway for lipid A production, such as the *firA*⁻ mutation (Hirvas et al., 1991, EMBO J. 10:1017-1023) and one or more mutations to auxotrophy for one or more nutrients or metabolites, such as uracil biosynthesis, purine biosynthesis, and arginine biosynthesis as described by Bochner, 1980, J. Bacteriol. 143:926-933. In a more prefered embodiment of the invention, the bacterial vector which selectively targets tumors and expresses a pro-drug converting enzyme is auxotrophic for uracil, aromatic amino acids, isoleucine and valine and synthesizes an altered lipid A.

6.3. *IN VITRO* CANCER DIAGNOSTICS AND *IN VIVO* TREATMENT OF SOLID TUMORS USING ISOLATED VECTORS AND OTHER VECTORS

6.3.1. *IN VITRO* DIAGNOSTICS

An embodiment of the present invention is to provide methods for use of the vectors of the present invention in *in vitro* diagnostic assays and diagnostic kits for the detection of solid tumor cancers, including but not limited to melanoma. Also, the kits may comprise tumor-specific non-attenuated vectors. The *in vitro* diagnostic assays and kits are based on the enhanced specificity towards

a cancerous cell rather than its non-cancerous counterpart cell of a vector. For example, and not by way of limitation, a putative solid tumor is biopsied from a patient. The tumor biopsy is minced and digested to a suspension of single cells. Aliquots of the suspension and a non-cancerous counterpart or control cell are cultured and infected with a tumor-specific vector according to the present invention.

After an incubation period, the number of tumor-specific microorganisms which attached to and/or infected the biopsied cells as compared to the non-cancerous counterpart or control cells is determined by any method known to those skilled in the art. A higher number of vectors found associated with the target cell as compared to the non-cancerous counterpart or control cells indicates that the target cell is cancerous, for example, about 5-10 times as many vectors will infect a tumor cell compared to a non-cancerous control cell. A non-cancerous counterpart or control cell is the normal cell from which the tumor cell is derived, for example, for melanoma cells the non-cancerous counterpart or control cell is melanocyte cells, for colon cancer the counterpart cell is colon epithelial cells. In one embodiment the ratio is determined as the number of vectors/target cell. In another embodiment, after infection, the cells are fixed and treated with a stain or antibody which recognizes DNA so that the vector DNA present in the target cell cytoplasm is visualized. The presence of DNA in the target cell cytoplasm indicates that the biopsied target cells are cancerous. In one embodiment of the present invention the diagnostic method comprises exposing a sample of cells which are suspected of being cancer cells to a tumor-specific vector or microorganism. The method also comprises exposing a sample of non-cancerous counterpart cells to the tumor-specific vector or microorganism as a comparative control. After incubating for a time period in which the microorganisms can attach to and/or infect cancer cells, the infectivity of the microorganism or vector for the

cells suspected of being cancerous and the non-cancerous counterpart control cells can be compared.

The diagnostic kits of the present invention comprise an effective amount of a tumor-specific vectors. The kits can further comprise an appropriate amount of non-cancerous control cells. The vector and/or cells may be supplied either frozen, lyophilized or growing on solid or in liquid medium. The diagnostic kits can further comprise inert ingredients and other kit components such as vials, packaging components and the like, which are well known to those skilled in the art.

In certain embodiments, the vectors useful for the methods of diagnosis of the present invention can further comprise tumor-specific, attenuated or non-attenuated vectors. In other embodiments, the kits of the present invention can comprise tumor-specific, attenuated or non-attenuated vectors.

For illustrative examples of in vitro diagnostics of solid tumor cancers, including but not limited to melanoma, see Sections 22, 25 and 26.

6.3.2. *IN VIVO* TREATMENT OF SOLID TUMORS

The vectors for use in *in vivo* cancer treatment are a subset of the vectors of the present invention. The vectors for *in vivo* treatment have been attenuated such that, when administered to a host, the vector has been made less toxic to the host and easier to eradicate from the host's system. In a preferred embodiment, the vectors are super-infective, attenuated and specific for a target tumor cell. In a more preferred embodiment, the vectors are also sensitive to a broad range of antibiotics.

In addition, the isolated vectors can encode "suicide genes", such as pro-drug converting enzymes or other genes, which are expressed and secreted by the vector in or near the target tumor. The gene can be under the control of either constitutive, inducible or cell-type specific promoters. In a preferred embodiment, a suicide gene is

expressed and secreted only when a vector has invaded the cytoplasm of the target tumor cell, thereby limiting the effects due to expression of the suicide gene to the target site of the tumor.

In a preferred embodiment, the vector, administered to the host, expresses the *HSV* TK gene. Upon concurrent expression of the TK gene and administration of ganciclovir to the host, the ganciclovir is phosphorylated in the periplasm of the microorganism which is freely permeable to nucleotide triphosphates. The phosphorylated ganciclovir, a toxic false DNA precursor, readily passes out of the periplasm of the microorganism and into the cytoplasm and nucleus of the host cell where it incorporates into host cell DNA, thereby causing the death of the host cell.

Another embodiment of the present invention is to provide methods of treatment of solid tumor cancers with isolated attenuated vectors of the present invention. For example, a patient is diagnosed with a solid tumor cancer by any method known in the art, including the *in vitro* diagnostic methods of the present invention. The vector used in the treatment may already be isolated using the methods of the present invention with target cell lines or using model tumors in mice of the target tissue. In another embodiment, the biopsied tumor cells are used in the selection assay for isolating a vector which is super-infective and tumor-specific for the tumor of the patient. In a preferred embodiment the vector is genetically modified, for example, to lack virulence factors, express a suicide gene or both as described in Section 6.2.2. In addition, the isolated vector is analyzed for sensitivity to antibiotics to insure the eradication of the vector from the patient's body after successful treatment or if the patient experiences complications due to the administration of the isolated vector.

When administered to a patient, *e.g.*, an animal for veterinary use or to a human for clinical use, the vectors can be used alone or may be combined with any physiological

carrier such as water, an aqueous solution, normal saline, or other physiologically acceptable excipient. In general, the dosage would range from about 1 to $1x10^8$ c.f.u./kg, preferably about 1 to $1x10^2$/kg.

The vectors of the present invention can be administered by a number of routes, including but not limited to: orally, topically, injection including, but limited to intravenously, intraperitoneally, subcutaneously, intramuscularly, intratumorally, *i.e.*, direct injection into the tumor, etc.

The following series of examples are presented by way of illustration and not by way of limitation on the scope of the invention.

7. EXAMPLE: ISOLATION OF SUPER-INFECTIVE, TUMOR-SPECIFIC *SALMONELLA TYPHIMURIUM IN VITRO*

7.1. MUTAGENESIS BEFORE ISOLATION OF SUPER-INFECTIVE, TUMOR-SPECIFIC CLONES

A culture of *Salmonella typhimurium* strain #14028 was grown exponentially at 37°C in minimal medium 56 plus glycerol (0.5%) to OD^{600}= 0.3, then chilled on ice. An aliquot was removed so that the culture could be titered for colony forming units (c.f.u.) on LB agar plates. The culture was washed and resuspended in Na citrate (0.1M, pH 5.5), incubated with fresh nitrosoguanidine (NG, 50μg/ml, 20 minutes, 37°C), washed once by centrifugation, resuspended in medium 56, chilled, and again an aliquot was removed so that the culture could be titered for c.f.u. on LB agar plates. Another aliquot of the NG treated bacteria was diluted (1:5) into LB broth and grown to stationary phase for storage frozen at -80°C in 12% glycerol.

The remaining bacteria were irradiated with ultraviolet light, dose = 50J/m2, λ= 254nm). An aliquot was removed and the cells were then titered for c.f.u. on LB agar plates, with another aliquot diluted 1:4 into LB broth, grown to stationary phase, and stored frozen at -80°C in 12% glycerol.

The mutagenesis procedures produced an increase in the number of mutations in the strain by four criteria: 1) decreased survival of the bacteria following mutagenesis (nitrosoguanidine=6-fold; ultraviolet B irradiation=400-fold); 2) increased frequency of auxotrophic (nutritional requiring) mutants to (2%); 3) increased frequency of maltose$^-$ mutants to (2%); 4) increased frequency of galactose$^-$ mutants to (0.5%).

7.2. ISOLATION OF SUPER-INFECTIVE *SALMONELLA TYPHIMURIUM* CLONES #70 AND #71 SPECIFIC FOR CANCER CELLS

A population of *Salmonella typhimurium* wild type strain #14028 was mutagenized as described in Section 7.1 with nitrosoguanidine and UV irradiation. Briefly, the bacteria were grown exponentially at 37°C in minimal medium 56 plus glycerol to OD^{600}=0.3, chilled on ice, washed, resuspended in Na citrate with 50μg/ml nitrosoguanidine and incubated for 20 minutes at 37°C. The bacteria were washed once by centrifugation and resuspended in medium 56. The bacteria were then irradiated with UV light at a dose of 50J/m^2, λ=254nm.

Prior to infection by *Salmonella*, human M2 melanoma cells were inoculated into Coming Tissue Culture flasks (25cm^2) at approximately 2x10^5 cells/flask in 4 ml DMEM cell culture medium containing penicillin (100 units/ml), and streptomycin (100μg/ml), and incubated overnight in a 37°C, gassed (5%CO2), humidified incubator. The next day the cells were rinsed twice with prewarmed Dulbecco's Minimal Essential medium supplemented with 10% fetal bovine serum (DMEM/FBS) and no antibiotics.

The mutated population of *Salmonella typhimurium* was cultured on LB agar overnight at 37°C or in a liquid culture. The following day the bacteria were transferred with a platinum wire loop to LB broth or to DMEM/FBS, adjusted in concentration to OD^{600}= 0.1 (approximately 2 x 10^8 c.f.u./ml), and subjected to further growth at 37°C on a

rotator. Following growth to the desired population density (monitored at an optical density of 600nm) the bacteria were diluted to a concentration of 10^6 c.f.u./ml in DMEM/FBS, and incubated at 37°C an additional 20 minutes.

The mutagenized bacterial population was subjected to a single cycle of infection into- and isolation from human M2 melanoma cells in culture. Portions of the mutagenized population were grown clonally on agar and 20 clones of *Salmonella typhimurium* were separately isolated and tested for their individual infectivity toward human M2 melanoma cells. The bacteria were added to animal cell cultures in 25 cm^2 Coming Tissue Culture flasks at 4ml/flask, and incubated with the animal cells in a gassed (5%CO^2/95% air), humidified incubator at 37°C. After a 15 minute incubation with the animal cells the bacteria-containing medium was poured off and the cultures were rinsed gently with warmed DMEM/FBS (4 ml) containing gentamicin sulfate (20μg/ml), an antibiotic that kills extracellular but not intracellular bacteria. The gentamicin sulfate-containing medium was poured off, fresh DMEM/FBS/gentamicin sulfate medium was added, and the cells were incubated for 60 minutes at 37°C. Following the 60 minute incubation with gentamicin sulfate, the medium was poured off, the flasks were rinsed 1x with DMEM/FBS (without gentamicin sulfate), and 1 mM EDTA or an EDTA/trypsin solution (Sigma Chemicals, 1x) in Ca^{++}/Mg^{++} free physiological saline (4 ml) was added. After incubating with EDTA or EDTA/trypsin for 20 minutes at 37°C, the flasks were shaken to suspend the animal cells, and aliquots were removed for quantitation. Animal cells were quantitated in a Coulter Counter (Coulter Electronics, Inc.) and bacteria were quantitated by plating aliquots on LB agar, incubating at 37°C, and counting colonies. Quantitation was expressed as the number of infecting (gentamicin resistant) bacteria/10^6 animal cells.

Two clones, "70" and "71", were found to be super-infective of melanoma cells, with infection capacities 5-10-fold greater than the mutagenized wild type strain (data not

shown). Clones 70 and 71 were also assessed for their relative specificity of the following human cells in culture: M2 melanoma cells and normal human melanocytes; "CaCo" colon cancer cells and normal human colon epithelium #1790 as depicted in Table 4(A).

TABLE 4(A)

SPECIFIC INVASION OF *S. TYPHIMURIUM* INTO MELANOMA VS MELANOCYTES AND COLON CANCER VS COLON EPITHELIUM IN CELL CULTURE: CLONES "70" AND "71"+

Human Cell Line	Infecting Salmonella/10^6 human cells: Clone 70	(ratio)*	Clone 71	(ratio)*
normal melanocytes	1.4 ± 0.2x10^6		1.2 ± 0.3x10^6	
M2 melanoma	7.3 ± 2.0x10^6	(5.2)	5.7 ± 0.7x10^6	(4.8)
colon epithelium (#1790)	1.5 ± 0.1x10^6		0.8 ± 0.2x10^6	
colon carcinoma (CaCo)	7.2 ± 2.0x10^6	(4.8)	2.3 ± 0.3x10^6	(2.9)

*cancer cell:normal counterpart cell
+Results represent averages ± SD for triplicate infections.

The bacterial clones #70 and #71 showed strong invasion preference for melanoma and colon cancer cells over that for normal melanocytes and normal colon epithelial cells.

7.3. ISOLATION OF *SALMONELLA TYPHIMURIUM* SUPER-INFECTIVE CLONE #72 BY CYCLING IN *IN VITRO* CELL CULTURE

Salmonella wild type strain #14028 was mutagenized with nitrosoguanidine and ultraviolet B irradiation as described in Section 7.1. A starting population of 5 x 10^8 mutagenized bacteria was grown to OD^{600}=.450, diluted in

DMEM/FBS to a concentration of 5 x 10^7 c.f.u./ml, and allowed to infect human M2 melanoma cells for 15 minutes. Infecting bacteria were isolated from the melanoma cells, and again allowed to infect fresh, uninfected populations of melanoma cells. The 2nd round of infecting bacteria were again isolated and subjected to further cycles of infection into, and isolation from, human M2 melanoma cells. After the completion of 4 such cycles, the population of melanoma-cycled bacteria which is designated 14028^{pop-1} was then plated on agar and 100 individual clones were picked and tested for their ability, compared to wild type bacteria, to infect M2 melanoma cells. The results of the selection process on 14028^{pop-1} and selected population sub-clones are detailed in Table 5.

Additionally, an aliquot of 14028^{pop-1} was subjected to two further cyclings in M2 melanoma cells. This 6X-cycled population was then subjected to 7 cycles of negative selection against normal human melanocytes. The 6X-cycled population was added to a culture of normal human melanocytes and incubated for 15 minutes. The supernatant was collected and was then added back to a fresh culture of normal human melanocytes. This negative selection procedure was carried out 7 times. This 6X-7X cycled population was again added to M2 melanoma cells, allowed to infect the melanoma cells for 15 minutes, and the bacteria were then collected from the M2 cells. This 6X-7X-1X cycled population was designated 14028^{pop-2}.

The mixed population of 4 times cycled *Salmonella typhimurium*, designated 14028^{pop-1}, showed a 3-fold increased infectivity of melanoma cells over that of the starting mutagenized population of wild type bacteria. Of the 100 clones isolated from population 14028^{pop-1} of *Salmonella*, two clones, #6 and #72, were found to be significantly super-infective of melanoma cells. The remaining bacterial clones showed infectivity that was similar to or below that of the wild type strain. In the experiment presented in Table 5, clone #6 was about 25-fold, and clone #72 was about 55-fold

more infective than the mutagenized wild type strain during a 15 minute infection period. *Escherichia coli*, strain K-12, #CSH 101, was at least two orders of magnitude less infective than wild type *Salmonella typhimurium*, thus, demonstrating the natural ability of *S. typhimurium* to infect certain animal cells.

TABLE 5

INFECTION OF M2 HUMAN MELANOMA CELLS WITH VARIOUS ISOLATED *SALMONELLA TYPHIMURIUM* POPULATIONS IN CULTURE+

Salmonella Strain	Infecting Bacteria/10^6	melanoma cells (%wild type)
Wild type *S. typhimurium* #14028 (mutagenized)	$3.8 \pm 3.0 \times 10^4$	100
#14028^{pop-1}	$1.1 \pm 0.4 \times 10^5$	290
Clone #6	$8.6 \pm 1.0 \times 10^6$	2260
Clone #72	$2.1 \pm 0.2 \times 10^6$	5500
E. coli K-12	$<10^2$	<1

+Results represent averages ± SD for triplicate infections.

Over several such experiments shown in Table 5, the infectivity of clone #72 toward melanoma cells varied from 5- to 90-fold over that of the wild type strain. This variation seemed to depend on the bacterial growth density prior to infection of melanoma cells. Therefore, the effect of population density on relative infectivity between wild type and clone #72 was determined.

Wild type *S. typhimurium* and super-infective clone #72 were grown as a lawn on LB agar plates. Portions of the cultures were removed with a platinum loop and inoculated into LB broth at a concentration of approximately 2×10^8 c.f.u./ml (OD^{600}=0.1). The cultures were then placed on a rotator at 37°C and optical densities were monitored as a function of population density. At the optical densities

indicated, aliquots of bacteria were removed, diluted in melanoma growth medium (DMEM/10% FBS) to a density of 1 x 10^6 c.f.u./ml, and allowed to infect human M2 melanoma cells. Infectivity assays were carried out as described. The results are shown in Table 6.

TABLE 6

INFECTIVITY OF WILD TYPE *S. TYPHIMURIUM* AND SUPER-INFECTIVE CLONE #72 TOWARDS HUMAN MELANOMA CELLS: EFFECT OF BACTERIAL POPULATION DENSITY+

Optical Density: *Salmonella*/10^6 melanoma			Infectivity ratio:
(600nm)	Clone #72	Wild type	(Clone 72:wild type)
0.200	-0-	-0-	(no infectivity)
0.300	9.0 x 10^3	-0-	(infinite)
0.400	5.0 x 10^4	-0-	(infinite)
0.500	4.5 x 10^5	5.0 x 10^3	90:1
0.600	1.2 x 10^6	3.7 x 10^4	32:1
0.700	2.3 x 10^6	2.5 x 10^5	9:1
0.800	3.2 x 10^6	5.6 x 10^5	6:1
0.900	3.6 x 10^6	7.3 x 10^5	5:1

+Results represent averages of duplicate experiments. Variations between duplicates were < ± 15%.

The results demonstrate that infectivity of both bacterial strains was highly dependent on bacterial population density prior to infection, however, clone #72 was proportionately more infective than the wild type strain at low population densities.

The results shown in Tables 5 and 6 were also confirmed by phase and light microscopy which revealed super-infectivity of a 10x melanoma-cycled population of *Salmonella typhimurium* designated "M10" as shown in Figures 2 and 3. It was also found that wild type strain 14028 and clone 72 infected human M2 melanoma cells equally well when grown

under anaerobic conditions prior to infection. However, when the strains are grown under aerobic conditions, strain 14028 was strongly suppressed in infectivity, whereas clone 72 remained induced. Thus, clone 72 was infective under either anaerobic or aerobic growth conditions and superinfective compared to wild type under aerobic growth conditions.

7.4. PREFERENTIAL SELECTIVITY OF *S. TYPHIMURIUM* FOR CANCER CELLS: WILD TYPE STRAIN VS. SUPER-INFECTIVE CLONE #72

Super-infective *Salmonella typhimurium* clone #72 isolated in Section 7.3 was compared to the non-mutagenized wild type strain #14028 for relative infectivity of human M2 melanoma cells, normal human melanocytes, colon cancer cells and normal colon epithelium. The results are shown in Table 7.

TABLE 7

TUMOR SPECIFICITY OF WILD TYPE *S. TYPHIMURIUM* AND SUPER-INFECTIVE CLONE #72 TOWARD VARIOUS NORMAL AND CANCEROUS CELLS IN CULTURE+

Animal	Infecting *Salmonella*/ 10^6 animal cells			
Cell Line	wild type	(ratio)*	Clone #72	(ratio)*
normal melanocyte (foreskin, human)	$1.2 \pm 0.7 \times 10^4$		$2.7 \pm 0.4 \times 10^5$	
M2 human melanoma	$2.5 \pm 0.6 \times 10^4$	(2.1)	$1.7 \pm 1.1 \times 10^6$	(6.3)
normal colon epithelium (1790, human)	$6.6 \pm 0.8 \times 10^3$		$5.2 \pm 3.0 \times 10^5$	
colon cancer (HTB 39, human)	$3.0 \pm 2.0 \times 10^4$	(4.6)	$9.5 \pm 3.0 \times 10^5$	(1.8)
"normal" fibroblast (3T3, mouse)	$1.8 \pm 1.5 \times 10^4$		$5.5 \pm 1.4 \times 10^5$	
transformed macrophage (J774, mouse)	$2.4 \pm 0.6 \times 10^4$	(1.3)	$4.6 \pm 0.8 \times 10^6$	(8.4)

+Results represent averages ± SD for triplicate infections.
*cancer cell:normal counterpart cell

Each of the two bacterial strains showed invasion preference for human cancer cells over normal cells. Clone #72 was super-infective in all cases when compared to the wild type strain. Further, clone #72 showed a significantly higher degree of invasion specificity for human melanoma cells over normal melanocytes than the wild type strain did.

7.5. INFECTIVITY OF *SALMONELLA TYPHIMURIUM* WILD TYPE STRAIN 14028 AND SUPERINFECTIVE CLONE 72 TOWARD VARIOUS HUMAN CARCINOMAS IN CULTURE

In another series of experiments, the relative infectivities of clone 72 and wild type strain 14028, toward a variety of human carcinomas growing in culture, was determined. The experimental protocol used is described in Section 7.2. Results are presented in Table 7(A).

TABLE 7(A)

INFECTIVITY OF *SALMONELLA TYPHIMURIUM* WILD TYPE AND SUPERINFECTIVE CLONE 72 TOWARD VARIOUS HUMAN CARCINOMAS IN CULTURE

	Salmonella/10^6 Human Cells			
Cell Line	Origin of Primary Tumor	Wildtype: 14028	Clone # 72	Ratio 72:14028
M2	melanoma	$4.0 \pm 3.8 \times 10^4$	$4.2 \pm 3.5 \times 10^5$	11:1
HTB57	lung	$2.8 \pm 1.3 \times 10^3$	$4.5 \pm 2.1 \times 10^4$	16:1
HTB183	lung	$1.0 \pm 0.3 \times 10^5$	$4.1 \pm 1.8 \times 10^5$	4:1
HTB54	lung	$2.1 \pm 0.7 \times 10^4$	$1.7 \pm 0.2 \times 10^5$	8:1
A549	lung	$3.7 \pm 5.6 \times 10^4$	$4.5 \pm 4.9 \times 10^5$	12:1
CRL1740	prostate	$2.3 \pm 0.4 \times 10^5$	$1.8 \pm 0.2 \times 10^6$	8:1
CRL1611	kidney	$3.2 \pm 1.2 \times 10^5$	$1.8 \pm 0.3 \times 10^5$	6:1
HTB52	liver	$1.8 \pm 0.3 \times 10^5$	$2.6 \pm 0.8 \times 10^5$	1.4:1
MCF7	breast	$7.3 \pm 2.6 \times 10^4$	$3.6 \pm 0.9 \times 10^5$	5:1

Results represent averages ± SD for n = 3-9 separate infections.

Both the wild type strain 14028 and clone #72 were able to infect each of the human cancer cells tested in culture. In all cases, clone 72 was superinfective compared to the wild type strain.

However, human lung line HTB57 was significantly less receptive to *Salmonella typhimurium* infectivity when compared to other cancer cell lines tested. In yet another

series of experiments, the human lung line HTB57 was implanted into mice. In 10 of 10 *nu/nu* mice implanted with 1 x 10^7 HTB57 cells, no tumor "takes" were observed, even after several months. Whether or not these cells were receptive to *Salmonella* infection when grown as tumors was not determined.

7.6. **DISCUSSION**

In summary, the results demonstrate the following: a) infectivity of *S. typhimurium* is dependent upon population density of the bacteria and b) super-infective clone #72 differs from the wild type strain in its increased infectivity of melanoma cells at all bacterial population densities and especially at low population densities under aerobic growth conditions. The ability to infect at low bacterial population densities is an advantage in the use of clone #72 as a tumor-specific vector, since it would allow for a lower c.f.u. of bacteria inoculated into the cancer patient, thus reducing the risk of septic shock in the patient. Additionally, the results demonstrate methods for the isolation of super-infective, tumor-specific mutants of *S. typhimurium*. Such mutants are represented by clones #6, #70, #71 and #72 that were isolated via enrichment procedures for melanoma infectivity by the bacteria. The results further demonstrate that wild type *S. typhimurium* exhibits specificity for human cancer cells over normal human cells in culture. Further, although clone 72 was originally selected for superinfectivity toward human melanoma cell line M2, it was additionally found to be superinfective toward human colon cancer cells and transformed mouse macrophages, when compared to the wild type strain 14028 (*see* Table 7). The expression of super-infectivity and tumor-specificity of isolated mutant clones represent attenuation of the bacteria and present distinct advantages for the use of such *Salmonella* clones as tumor-specific vectors in the diagnosis and therapy of human cancer.

8. **EXAMPLE: SELECTION FOR *SALMONELLA TYPHIMURIUM* MUTANTS WITH CHEMOTACTIC ABILITIES TOWARD MELANOMA SECRETORY PRODUCTS *IN VITRO***

The melanoma cells were an artificially-produced hybrid line isolated from a polyethylene glycol induced fusion between Cloudman S91 mouse melanoma cells and peritoneal macrophages from a DBA/2J mouse. The hybrid cell line used herein was termed Cloudman S91 melanoma/macrophage hybrid #48. The hybrid cell line formed rapidly growing metastasizing tumors in DBA/2J mice, Pawelek et al., 1995, J. Invest. Dermatol. 104:605. 5 x 10^6 Cloudman S91 melanoma/macrophage hybrid #48 cells were cultured at 30°C in a gassed, humidified incubator in 75cm^2 culture flasks in DMEM/FBS culture medium containing 10% fetal bovine serum and no antibiotics. Control flasks containing DMEM/FBS but no melanoma cells were incubated in parallel. After 72 hours, the media were removed, aseptically filtered through 0.45μ filters, and stored at 4°C.

Salmonella typhimurium super-infective clone #72 described above was subjected to mutagenesis with nitrosoguanidine and UV. The mutagenesis procedures produced an increase in the number of mutations in Clone #72 similar to that shown earlier when the wild type strain #14028 was mutagenized. This mutagenized population of clone 72 ("72^{mut}") was further used to select for mutants with enhanced chemotactic abilities toward melanoma cell secretory products, *i.e.*, melanoma-conditioned culture media.

Procedures for loading capillary tubes with potential chemotactic attractants were modified from Adler (Adler, 1973, J. General Microbiology 74:77-91). Control and melanoma-conditioned culture media, described above, were loaded into 2λ capillary tubes ("Microcaps", Drummond Scientific Co.) as described by Adler. The capillaries were handled with forceps. One end was sealed in a flame; the capillary was then quickly passed several times through the flame and immediately plunged open end down into a 10 ml beaker containing 1 ml control or melanoma conditioned

culture medium. As the capillary cooled (about 10 minutes), liquid was drawn in about 1 cm.

Salmonella typhimurium, growing at 37°C in LB were collected by centrifugation and resuspended in control DMEM/FBS culture medium containing a concentration of 10^8 c.f.u./ml. Aliquots (200 µl, 2 x 10^7 c.f.u.) were pipetted into 1.5 ml microfuge tubes. Loaded capillary tubes (described above) were then inserted open end down into the Beckman microfuge tubes containing the Salmonella typhimurium, and the assay was begun by incubating at 37°C. After 30 to 60 minutes, the capillary tubes were removed with forceps, the sealed ends were broken off with wire cutters, and the capillaries were transferred to 15 ml conical centrifuge tubes containing 3 ml LB broth. It was important that the upper tips of capillary tubes were covered with LB broth in order to assure quantitative recovery of the bacteria via the centrifugation step described as follows. The capillaries within the centrifuge tubes were then centrifuged (1000x g for 4 minutes) to force the bacteria out of the capillaries. The bacteria were resuspended by vortexing, and aliquots were spread onto LB agar plates for quantitation. Significant increases in the number of bacteria entering the capillaries containing melanoma-conditioned media compared to control-conditioned media indicated a chemotactic response of the bacteria to melanoma-secreted products.

Aliquots of mutagenized super-infective *Salmonella typhimurium*, "72^{mut}", described above were placed on a rotor at 37°C, grown to an optical density of 0.4-0.6 at a wavelength of 600nm, and subjected to the chemotaxis procedures described above. The chemotaxis cycling procedure was repeated 4 times through successive challenges with melanoma-conditioned culture medium. The population obtained after 4 cycles was designated #72^{pop-2}. After the 4th cycling, aliquots of the mixed populations of bacteria were frozen in glycerol. Additional aliquots of the mixed population of *Salmonella typhimurium* obtained from the fourth cycling were

then compared to an uncycled mixed population of mutagenized clone 72 ("72^{mut}") for relative chemotactic abilities toward control and melanoma-conditioned culture medium. The results are shown in Table 8.

TABLE 8

EVIDENCE FOR POSITIVE CHEMOTACTIC RESPONSES OF *S. TYPHIMURIUM* TO CONDITIONED GROWTH MEDIUM OF CULTURED MELANOMA CELLS+

Salmonella Strain	Bacteria/Capillary Tube: Control Medium	Conditioned Medium	Ratio:
$\#72^{mut}$ (mutagenized, no cycling)	$1.2x10^3 \pm 0.2$	$4.4x10^3 \pm 2.7$	3.7:1
$\#72^{pop-2}$ (mutagenized, cycled 4x)	$0.5x10^3 \pm 0.1$	$1.8x10^3 \pm 0.4$	3.6:1

+Results represent average ± S.D for quadruplicate capillary tubes.

Both populations of bacteria tested showed positive chemotactic responses to melanoma-conditioned culture medium over control-conditioned medium, displaying an approximate 4:1 preference for the melanoma-conditioned medium. Although the chemotactic response of population $\#72^{pop-2}$ was not statistically significant as compared to the chemotactic response of population $\#72^{mut}$ for melanoma conditioned medium, the chemotactic response of population $\#72^{pop-2}$ was significantly reduced as compared to the chemotactic response of population $\#72^{mut}$ for control medium. Thus, the propensity of population $\#72^{pop-2}$ to enter capillary tubes containing control medium was significantly reduced. These results suggest that population $\#72^{pop-2}$ is less efficient in motility generally, however, upon exposure to melanoma-conditioned medium, population $\#72^{pop-2}$ showed a chemotactic response equivalent to the control population.

Whatever the mechanisms for the different chemotactic phenotypes expressed by the #72^{mut} and #72^{pop-2} populations of bacteria in Table 8, the results demonstrate that the phenotypes can be altered via the selection procedure of exposing bacteria to successive challenges of melanoma-conditioned media. It is likely that the mixed populations of mutagenized, chemotactically cycled bacteria isolated in these experiments contain a number of diverse mutants expressing likewise diverse phenotypes for the chemotactic response to melanoma cell-conditioned medium.

9. **EXAMPLE: ISOLATION OF TUMOR-SPECIFIC MUTANTS OF *SALMONELLA TYPHIMURIUM* BY CYCLING *IN VIVO* IN TUMOR-BEARING MICE**

Tumor cells inoculated into DBA/2J mice from Cloudman S91 melanoma/macrophage hybrid cell line #48 were used as the target tumor for the selection of attenuated, tumor-specific *Salmonella typhimurium*. Super-infective *Salmonella typhimurium* clone #72 was mutagenized with nitrosoguanidine and UVB as described in Section 7.1 producing a mutagenized population derived from clone #72. The mutagenesis procedures produced an increase in the number of mutations in clone #72 similar to that shown earlier when the wild type strain #14028 was mutagenized. Cloudman melanoma/macrophage hybrid #48 cells were inoculated (s.c.) into DBA/2J mice at a concentration of 10^6 cells in 0.1 ml saline/inoculated site and a total of 4 sites/mouse in the shoulder and flank regions. After 8-10 days, palpable tumors developed, and the mice were inoculated (i.p.) with the mutagenized *Salmonella* population derived from super-infective clone #72. After 2 hours of infection, the mice were sacrificed, the tumors removed, weighed, and homogenized in a teflon homogenizer in 5 vol (vol/wt) LB broth. An aliquot of the homogenate was then diluted about 1:4 in LB broth, placed on a rotator at 37°C, and incubated through 1-2 population doublings, should be monitored at OD^{600}, in order to ensure the recovery of viable bacteria for successive

inoculations into tumor-bearing mice. The procedure was repeated through 4 cycles of infection into mice, followed by recovery from tumors. At the beginning of each cycle, the number of bacteria inoculated and the time of infection was reduced from the previous cycle in order to increase the stringency of selection for tumor-specific mutants. The resultant population recovered after 4 cycles was designated #72^{pop-1}. The results of this procedure are detailed in Table 9 below.

TABLE 9

SELECTION FOR MELANOMA-SPECIFIC *SALMONELLA TYPHIMURIUM* IN TUMOR-BEARING MICE

Infection Cycle	Total # Bacteria Inoculated/mouse	Infection Time	Total # Bacteria Recovered in Tumors*
1	$1x10^{10}$	120 min	2.1×10^7
2	$1x10^9$	80 min	1.6×10^6
3	$6x10^8$	60 min	1.7×10^8
4	$2x10^8$	40 min	1.4×10^5

*Infecting *Salmonella* were pooled from 4-8 separate tumors for each cycle

These results demonstrate that infecting bacteria can be recovered from tumors *in vivo*. These results also demonstrate that *in vivo* cycling results in an enriched population since fewer bacteria were isolated than were inoculated.

10. **EXAMPLE: PROLIFERATION OF *SALMONELLA TYPHIMURIUM* WITHIN MELANOMA CELLS**

Proliferation of a gene-delivering vector within target tissue can both amplify the gene within the target tissue as well as allow one to reduce the titer of inoculated vector, thus reducing the risk of septic shock in the host. The following examples demonstrate that *Salmonella typhimurium* proliferates in melanoma cells.

10.1. PROLIFERATION WITHIN CULTURED HUMAN M2 MELANOMA CELLS

It was found that *Salmonella typhimurium* proliferated within human M2 melanoma cells in culture with doubling times of about 30 to 60 minutes as illustrated below. Wild type *Salmonella* strain #14028 and super-infective clone #72 were separately introduced into the culture media of human M2 melanoma cells 2 x 10^5 melanoma cells/25cm^2 tissue culture flask at 10^6 bacterial c.f.u./ml culture medium. After 1 hour, gentamicin (20 μg/ml) was added to kill external, but not internalized bacteria, and melanoma cells were harvested and assayed for the number of internalized bacteria at the time points indicated. The results are presented in Table 10.

TABLE 10

PROLIFERATION OF *SALMONELLA TYPHIMURIUM* WILD TYPE STRAIN #14028 AND CLONE #72 WITHIN CULTURED HUMAN M2 MELANOMA CELLS+

Salmonella Strain	Time (h)	*Salmonella*/10^6 Melanoma Cells	Fold Increase
#14028 wild type	1	6.8×10^5	---
	2	1.8×10^6	2.6x
	4	1.8×10^7	26x
	6	5.4×10^7	79x
Clone #72	1	5.8×10^6	---
	2	8.0×10^6	1.4x
	4	3.2×10^7	5.5x
	6	1.4×10^8	24x

+The numbers represent averages for duplicate and triplicate determinations, with the variation between replicates < ± 25%.

10.2. PROLIFERATION WITHIN MELANOMA TUMORS GROWN IN MICE

DBA/2J mice were inoculated s.c. in four areas (left and right shoulders and flanks) with 10^6 Cloudman S91 melanoma/macrophage hybrid #48 cells. After the appearance of palpable tumors (8-10 days) the mice were further inoculated (i.p.) with 2 x 10^8 *Salmonella typhimurium*. The *Salmonella* strains tested were wild type #14028 and super-infective clone #72. At 4 hours and 21 hours post-inoculation with bacteria, mice were bled orbitally, and then euthanized by anesthesia with metofane. Tumors and livers were removed aseptically, rinsed with sterile NaCl (0.9%), weighed, and homogenized in LB broth at a ratio of 5:1 (vol:tumor wt). Bacteria were quantitated by plating the homogenates onto LB plates, incubating overnight at 37°C, and counting bacterial colonies. Numbers represent averages ± S.D. The results for the 4 hour and 21 hour incubations of the bacteria in mice are detailed in Tables 11(A) and 11(B).

TABLE 11

A. DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 4 HOURS FOLLOWING INOCULATION (I.P.) INTO CLOUDMAN S91 MELANOMA-BEARING DBA/2J MICE

Salmonella Strain	*Salmonella*/ ml Blood	*Salmonella*/ /gm tumor (wet wt)	*Salmonella*/ gm liver (wet wt)	Tumor/ Liver
wild type	$6x10^5$	$8.9 \pm 2.5x10^4$(n=4)	$3.6x10^5$	1:4
clone 72	$2x10^5$	$3.5 \pm 3.3x10^4$(n=4)	$2.4x10^5$	1:7

B. DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 21 HOURS FOLLOWING INOCULATION (I.P.) INTO CLOUDMAN S91 MELANOMA-BEARING DBA/2J MICE

Salmonella Strain	*Salmonella*/ ml Blood	*Salmonella*/ gm tumor (wet wt)	*Salmonella*/ gm liver (wet wt)	Tumor/ Liver
wild type	$1.0x10^4$	$1.3 \pm 0.8x10^9$(n=4)	$4.4x10^6$	300:1
clone 72	$6.7x10^3$	$2.1 \pm 2.7x10^9$(n=4)	$5.2x10^5$	4000:1

At 4 hours post-inoculation of *Salmonella*, there were fewer bacteria in the tumors than in the blood stream and the liver for both wild type clone 14028 and clone 72. However, by 21 hours, *Salmonella* were found in great abundance in the tumors so that the ratio of bacteria/g tissue in tumors was 4,000:1 over that in the liver for super-infective mutant clone 72. After 21 hours post-inoculation of bacteria, the number of *Salmonella* in the tumors was similar for both the wild type *Salmonella* strain and clone 72, and was far greater than the total number of *Salmonella* originally inoculated, indicating that both wild-type and clone 72 strains of bacteria proliferated within the tumors. Thus, the ability of *Salmonella typhimurium* to infect melanoma cells and proliferate within them was expressed both in cell culture as seen in Table 10 and in tumors growing in mice as seen in Tables 11A and 11B.

The wild-type strain 14028 showed higher infectivity in liver than did clone 72. The higher infectivity of liver by the wild-type *Salmonella* was consistent with the observed greater lethality of the wild type stain toward DBA/2J mice than that produced by clone 72 at high bacterial inocula (>10^9 c.f.u./mouse, data not shown). Similar results were observed with C57BL\6J mice bearing B16F10 melanomas as seen in Table 18, Section 15.2. Together, these results demonstrate that selection for strains of bacteria or other parasites with enhanced tumor specificity in vitro yields mutant strains with attenuated host toxicity *in vivo*.

10.3. DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* IN TUMOR-BEARING MICE

The following experiments demonstrate that *Salmonella* can localize to and proliferate within a tumor of an animal bearing either multiply-implanted subcutaneous melanoma tumors or naturally occurring metastases.

10.3.1. **DISTRIBUTION OF *SALMONELLA* FOLLOWING DIRECT INOCULATION INTO CLOUDMAN S91 MELANOMA TUMORS**

DBA/2J mice were inoculated s.c. in four areas (left and right shoulders and flanks) with 10^6 Cloudman S91 melanoma/macrophage hybrid site. Palpable tumors appeared 8-10 days post-inoculation, representative animals were selected, and 2 of the 4 tumors (right shoulder and left flank) were directly inoculated with *Salmonella typhimurium* super-infective clone #72 at c.f.u.'s of 7×10^4 or 7×10^6 bacteria/tumor. At 21 hours post-inoculation, mice were euthanized with metofane. Tumors and livers were removed aseptically, rinsed with sterile NaCl (0.9%), weighed, and homogenized with NaCl at a ratio of 5:1 (vol:tumor wt). Bacteria were quantitated by plating the homogenates onto LB plates, incubating overnight at 37°C, and counting bacterial colonies. The results are shown in Table 12.

TABLE 12

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* CLONE 72 IN CLOUDMAN S91 MELANOMA-BEARING MICE 21 HOURS FOLLOWING DIRECT INOCULATIONS INTO TUMORS

Inoculum/ tumor	*Salmonella*/ g tumor (wet wt)	*Salmonella*/ g liver (wet wt)	Tumor/ Liver
7.2×10^4			
Tumor 1*	1×10^9	3.0×10^5	3,300:1
Tumor 2	3×10^7		100:1
Tumor 3	1×10^8		330:1
7.2×10^6			
Tumor 1*	4×10^9	5.0×10^6	800:1
Tumor 2	3×10^9		600:1
Tumor 3	3×10^7		6:1

*inoculated tumor

In summary, two days post-inoculation of super-infective *Salmonella typhimurium* clone #72 directly into specificized tumors, the *Salmonella* could be found in distal, non-inoculated tumors. The amounts of *Salmonella* found in the tumors far exceeded the amounts of *Salmonella* inoculated into the mice, proving that the *Salmonella* proliferated within the tumors. The results demonstrate that *Salmonella typhimurium* can proliferate within a tumor, exit that tumor via the circulatory system, travel to a distant tumor, and proliferate within that distant tumor.

10.3.2. **DISTRIBUTION OF *SALMONELLA* INTO CLOUDMAN S91 MELANOMA METASTASES**

This experiment shows that the bacteria should be able to target naturally-occurring metastases of solid tumors.

1 x 10^5 Cloudman S91 melanoma cells were inoculated s.c. in the tail of a DBA/2J mouse. After approximately four weeks, a soft tissue metastasis (~0.5 g) developed with no visible evidence of a primary tumor in the tail. *S. typhimurium* clone 72 at 2 x 10^5 c.f.u. was inoculated i.p. The mouse was sacrificed 48 hours post- inoculation, and the liver and tumor were removed, homogenized in Luria broth, and quantitated for *S. typhimurium* by serial dilutions on LB agar plates.

The results shown in Table 12(A) demonstrate that *Salmonella typhimurium* clone 72 can target and proliferate within a metastatic tumor.

TABLE 12(A)

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* CLONE 72 IN A DBA/SJ MOUSE WITH A SOFT-TISSUE MELANOMA METASTASIS*

Tissue	Salmonella/g tissue	Tumor/Liver
Liver	3.1×10^6	---
Tumor	3.2×10^9	1000:1

*Results represent determinations from a single animal.

11. EXAMPLE: ANTIBIOTIC SENSITIVITY OF WILD TYPE *SALMONELLA TYPHIMURIUM* STRAIN 14028 AND SUPER-INFECTIVE MUTANT CLONE 72

11.1. SENSITIVITY TESTED *IN VITRO*

Wild type *Salmonella typhimurium* strain 14028 and super-infective mutant clone 72 were tested for antibiotic susceptibility and were each found to be sensitive to 12 different antibiotics currently used in treating bacterial infections. The bacteria were tested according to the standard protocol to determine antibiotic sensitivity as seen in clinical laboratories, so that a patient is not given an antibiotic to which the microorganism is resistant. The bacteria were tested for antibiotic susceptibility by subjecting them to the Disc Diffusion Susceptibility Technique Kit (Remel Corp., Lenexa, Kansas). The data are presented in Table 13.

TABLE 13

ANTIBIOTIC SENSITIVITY

	Wild Type:	Clone 72:
Ampicillin	S	S
Cefoperazone	S	S
Ceftazidime	S	S
Cefuroxime	S	S
Gentamicin	S	S
Mezlocillin	S	S
Cefazolin	S	S
Ciprofloxacin	S	S
Unasyn	S	S
Ceftriaxone	S	S
TMP/SMX	S	S

11.2. **SENSITIVITY TESTED *IN VIVO***

Susceptibility of *Salmonella typhimurium* clone 72 to antibiotics was further tested by injecting mice i.p. with bacteria, treating half of the mice with the antibiotic enrofloxacin, and observing the effects of enrofloxacin, an active analog of ciprofloxacin, on the survival of the mice.

Six week old C57B6 female mice were inoculated i.p. with 10^5 cfu *Salmonella typhimurium* clone 72. After four days following inoculation with bacteria, three of the mice were further inoculated i.p. with 100μg/0.1 ml BAYTRIL™ (enrofloxacin), and their drinking water was supplemented with 25μg/ml BAYTRIL™. After 4 days, all the mice were given fresh drinking water without BAYTRIL™. After a total of 21 days, all surviving mice were euthanized and the experiment was terminated. The results are shown below in Table 13(A).

TABLE 13(A)

SURVIVAL OF C57B6 MICE INJECTED WITH *SALMONELLA TYPHIMURIUM* CLONE 72 ± BAYTRIL® (enrofloxacin) IN DRINKING H_2O

Conditions	Avg. time of Death ± S.D.
no antibiotic	9.3 ± 5 days
enrofloxacin (days 4-10 post-inoculum)	> 21 days

Mice receiving bacteria only and no antibiotic died after an average of 9 days following inoculation. Mice receiving bacteria followed by antibiotic treatment survived at least 21 days and showed no symptoms of *Salmonella* toxicity when the experiment was terminated. Thus, the results clearly demonstrate that mice can be rescued from *Salmonella*-mediated death by treatment with the antibiotic enrofloxacin. These results are consistent with those presented in Table 13 demonstrating antibiotic sensitivity of *Salmonella typhimurium* strains 14028 and clone 72 by the Disc Diffusion Susceptibility Technique.

The results further underscore the advantage of using antibiotic-sensitive bacteria as vectors in human tumor therapy, since the bacteria can be eliminated by introduction of antibiotics when desired.

12. EXAMPLE: ENHANCED EXPRESSION OF BACTERIAL PROMOTERS IN MELANOMA CELLS

In a preferred embodiment of the present invention an isolated super-infective vector, such as *Salmonella typhimurium* clone 72^{5-3-2} which carries the *HSV* TK gene, the gene is specifically induced in cancerous target cells as opposed to normal cells in the host body. It has been shown that there is a higher relative induction of several

Salmonella promoter genes, including *pagB* and *pagC*, (Miller et al., 1989, Proc. Natl. Acad. Sci. USA 86:5054-5058; Miller et al., 1992, Infect. Immun. 60:3763-3770; Alpuche Aranda et al., 1992, Proc. Natl. Acad. Sci. USA 89:10079-10083) when the bacteria invade macrophages as opposed to epithelial cells. In order to test whether these promoters are also activated when *Salmonella* invade melanoma cells, we used *Salmonella*-bearing promoter constructs fused to the β-galactosidase reporter gene.

Human melanoma M2 cells, (Cunningham et al, 1992, Science, 255:325-327) human epithelial 1790 cells and mouse macrophage cell line J774 cells (American Type Culture Collection) were seeded at a density of 1 x 10^6 host cells in 25 cm^2 Corning tissue culture flasks. The cells were infected with 5 x 10^7 *Salmonella typhimurium* #14028/ml DMEM culture medium for 1 hour, washed with fresh medium, and further cultured for 6 hours with 50 μg/ml gentamicin added to the culture medium in order to kill the external but not the internalized *Salmonella*. The melanoma cells were then harvested by scraping them from the substratum in isotonic 1 mM EDTA solution. The cells were pelleted, resuspended in PBS and an aliquot was removed for quantitation of the bacteria found within the melanoma cells. The remainder of the melanoma cells were assayed for β-galactosidase activity.

Three *Salmonella typhimurium* clones were used: i) strain 14028 in which β-galactosidase was constitutively expressed; ii) strain 14028 in which β-galactosidase was expressed through activation of the *pagB* promoter; iii) strain 14028 in which β-galactosidase was expressed through activation of the *pagC* promoter. Thus, through measurements of β-galactosidase activity, analyses of bacterial *pagB* and *pagC* promoter induction in melanoma cells were carried out. The results are detailed in Table 14.

TABLE 14

ENHANCED EXPRESSION OF BACTERIAL PROMOTERS *PAGB* AND *PAGC* IN CULTURED HUMAN MELANOMA M2 CELLS*

	Promoter-Induced Activity:Constitute Activity	
Cell Line	*pagB*	*pagC*
human epithelial	1.3:1	7.2:1
mouse transformed macrophage	2.9:1	17:1
human melanoma	3.8:1	31:1

*relative activation of *pagB* and *pagC* was assessed through expression of promoter-inducible β-galactosidase activity.

Both the *pagB* and *pagC* *Salmonella* promoters were induced in human melanoma cells. Levels of induction in melanoma cells were greater than seen in either the epithelial or macrophage cell lines. These data demonstrate that the *pagB* or *pagC* promoter could be used to express genes, such as *HSV* TK or *E. coli* cytosine deaminase, in a melanoma cell-specific manner.

13. EXAMPLE: CLONING AND EXPRESSION OF PRODRUG CONVERTING ENZYMES

The following sections demonstrate useful systems for expression of prodrug-converting enzymes useful for the methods and compositions of the present invention.

13.1. CLONING AND EXPRESSION OF *HERPES SIMPLEX* VIRUS THYMIDINE KINASE IN *SALMONELLA TYPHIMURIUM*

Herpes simplex thymidine kinase (*HSV* TK) is known to be an effective pro-drug converting enzyme in the inhibition of melanoma tumor growth (Bonnekoh et al., 1995, J. Invest. Derm. 104:313-317). Accordingly, procedures were carried out to insert an *HSV* TK gene with the β-lactamase signal sequence into both *Salmonella typhimurium* wild type strain 14028 and super-infective tumor-specific mutant clone 72 which is derived from the wild type strain.

Herpes simplex thymidine kinase cloning by PCR

Plasmid DNA of the vector pHETK2 (Garapin et al., 1981, Proc. Natl. Acad. Sci. USA 78:815-819) was prepared by alkaline lysis, phenol/chloroform extraction and ethanol precipitation. PCR primers based on the complete sequence for the *Herpes simplex* thymidine kinase (McKnight, 1980, Nuc. A. Res. 8:5949-5964) were: forward 5'-GATCATGCATGGCTTCGTACCCCGGCC-3' (SEQ ID NO:1) and reverse 5'-CTAGATGCATCAGTGGCTATGGCAGGGC-3', (SEQ ID NO:2) which corresponds to bases 310-328 (forward) and 1684-1701 (reverse) of the published sequence, with an added sequence of GATCATGCAT (portion of SEQ ID NO:1) or CTAGATGCAT (portion of SEQ ID NO:2) (*Nsi*I site and spacer) at the 5'end of each primer. Each reaction mixture contained 50 ng DNA template, 10 pmoles of each primer, 100 mM deoxynucleotide triphosphates, 1.5 mM Mg^{++} and 0.5 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Amplification was performed by 35 cycles of 94°C for 1 minute; 50°C for 15 seconds; 55°C for 1 minute; and 72°C for 2 minutes. The amplified DNA was purified and was cloned into either pBluescript II KS+ and sequenced with T3 and T7 primers to confirm the correct DNA had been cloned or was cloned into p279 cut with *Pst*1 which provides the β-lactamase signal sequence (Talmadge et al., 1980, Proc. Natl. Acad. Sci. USA 77:3369-3373). Transformants were screened using a probe generated from the original template by random priming (Boehringer Mannheim, Indianapolis, IN) using [α-^{32}P]dCTP. Positive clones were further screened by immunoblot.

SDS-PAGE and Immunoblot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on bacterial lysates according to Weber and Osbom, 1975, Proteins and sodium dodecyl sulfate: Molecular mass determination on polyacrylamide gels and related procedures. In: H. Neurath and R. Hill (eds) The Proteins, Third Edition, vol. 1, Academic Press, New York pp. 179-223. Immunoblots were performed according to Towbin et al., 1979, Proc. Natl. Acad.

Sci. USA 76:4350-4354. Primary anti-TK antibodies were generally used at a 1:1000 dilution. Secondary anti-mouse antibodies were alkaline phosphatase-conjugates (Promega, Madison, WI) used at a 1:7,500 dilution, followed by nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl phosphate (BCIP) colorimetric detection (Promega).

Thymidine Kinase Assay

Bacterial lysates were prepared by pelleting 1 ml of log-phase bacterial culture for 30 seconds at 12,000 x g in a microfuge centrifuge. The pellet and supernatant were retained separately and the supernatant was further cleared by centrifugation for 10 min at 12,000 x g. The pellet was further treated by resuspension in 100 μl of phosphate buffered saline containing 1 mg/ml lysozyme and 1% (v/v) Triton X-100 and subjected to three cycles of rapid freezing and thawing. The resulting material was clarified by centrifugation at 12,000 x g for 2 minutes. Thymidine kinase activity was assayed using a modified version of the assay described by Summers and Summers, 1977, J. Virol. 24:314-318. The reaction mix was incubated at 37°C for 1 hour and then bound to DE81 paper (Whatman), washed, and the associated radioactivity determined in a gamma counter.

Salmonella transformation

Transformation of *Salmonella* strains was performed by electroporation as described by O'Callaghan and Charbit, 1990, Mol. Gen. Genet. 223:156-158. Plasmids transfected into *Salmonella* included pHETK2 (Garapin et al., 1981, Proc. Natl. Acad. Sci. USA 78:815-819) p279 (Talmadge et al., 1980, Proc. Natl. Acad. Sci. USA 77:3369-3373) and two independent isolates of β-lactamase fusions, p5-3 and p21A-2 (See Figure 4-C for a diagram of p5-3 and p21A-2 where these plasmids are designated "pTK Sec 1.". *Salmonella typhimurium* strains transfected were the wild type 14028 and the super-infective clone 72.

Two independent β-lactamase-TK gene fusion constructs were isolated and expressed in *Salmonella typhimurium* 14028 wild type and clone 72. An immunoblot

analysis and corresponding enzyme activity assay are presented in Figures 4A and 4B. All three TK-containing vectors, the cytoplasmically expressed pHETK2 and the β-lactamase fusions p5-3 and p21A-2, were detectable by immunoblot and enzyme assay. Relatively little enzyme activity was recovered from the culture supernatants. Since the immunoblot analysis shows processing of the signal sequence, secretion into the periplasmic space of the *Salmonella typhimurium* is expected.

13.2. SYSTEMS FOR EXPRESSION OF *HERPES SIMPLEX* THYMIDINE KINASE USING VARIOUS PROMOTERS AND SECRETION SIGNALS

A number of constructs were made to express TK using other promoters and other secretion signals.

13.2.1. EXPRESSION AS A *STAPHYLOCOCCUS* PROTEIN A FUSION UNDER THE LACI PROMOTER

Herpes simplex thymidine kinase was amplified by PCR as described in Section 13.1 above and cloned into the PstI site of pBluescript. This TK clone was subcloned from bluescript to the *Bam*HI and *Hind*III cite of the secretion vector pEZZ18 (Promega, Madison, WI; Nilsson and Abrahamsen, 1990, Methods in Enzymology 185:144-161). This resulted in an in-frame fusion with *Staphylococcus* protein A under the lacI promoter. This plasmid was designated pTK-Sec2 and is diagramed in Figure 4-C. Plasmid pTK-Sec2 expresses thymindine kinase as determined by an immunoblot.

13.2.2. CLONING OF THE SERRATIA MARCESENS CHITINASE SIGNAL SEQUENCE AND PROMOTER

The promoter and signal sequence of *Serratia marcesens* chitinase I (Jones et al., 1986, EMBO J. 5:467-473) was cloned by PCR. The forward and reverse primers had the following sequence: CTAGACTAGTTTGTCAATAATGACAACACCC (forward) (SEQ ID NO:3) and GATCGGATCCTTGCCCGGCGCGGCGGCCTG (reverse) (SEQ ID NO:4) which contain *Spe*I and *Bam*HI sites, respectively. The resulting product was cloned into pSP72

and confirmed by DNA sequencing. This plasmid was designated pSP-CHT and is also diagramed in Figure 4-C.

13.2.3. EXPRESSION AS A CHITINASE SIGNAL SEQUENCE FUSION UNDER THE CONTROL OF THE CHITINASE PROMOTER

Herpes simplex thymidine kinase in pBluescript was subcloned into the pSP-CHT vector using *Bam*HI and *Hind*III. This results in an in-frame fusion with the chitinase signal sequence under the chitinase promoter. This plasmid was designated pTK-Sec3 and is also diagramed in Figure 4-C. Plasmid pTK-Sec3 expresses thymindine kinase as determined by an immunoblot.

13.3. EXPRESSION OF P450 OXIDOREDUCTASE PRODRUG CONVERTING ENZYME IN BACTERIA USING AN EXOGENOUSLY INDUCIBLE PROMOTER

The *sulA* promoter element (GENBANK #V00358; Cole 1983) was cloned from *E. coli* genomic DNA by PCR using forward (CTAGAAGCTTATAAGGGTTGATCTTTGTTGTC) (SEQ ID NO:5) and reverse (GTACGATATCCAGAACGATGTGCATAGCCTG) (SEQ ID NO:6) primers which incorporate the *Hind*III and *Eco*RV restriction sites respectively. The PCR conditions were 35 cycles of 95°C, 1 minute; 55°C 1 minute; and 72°C for 1 minute. The product was cloned into pSP72 and sequenced with the T7 primer to confirm that the correct DNA had been obtained. The cloned DNA fragment was 100% identical to the published sequence.

The NADPH-dependent cytochrome p450 oxidoreductase (p450 OR) cDNA clone in the *Eco*RI site of pBluescript that lacks the first initiating ATG (deletion of the first 11 nucleotides) of the cDNA described by Yamano et al., 1989, Molecular Pharmacol. 35:83-88, was fused with the *sulA* promoter and initiating sequence by cloning the *sulA* promoter obtained as described above into the *Hind*III and *Eco*RV site of the p450 oxidoreductase gene. The resulting fusion consists of the *sulA* promoter, including the *sulA* ATG (methionine) and subsequent 9 amino acids (YTSGYAHRS) (SEQ ID

NO:7) as well as six amino acids which follow introduced from the DNA polylinker and PCR primers (SGYRIP) (SEQ ID NO:8) followed with the second amino acid of p450 OR, which is G. This construct, pSP-SAD4-5 is diagramed in Figure 4-D.

13.4. EFFECT OF EXPRESSION OF P450 OXIDOREDUCTASE CONVERSION OF PRODRUG ON BACTERIAL GROWTH

It has been previously shown (Shiba et al., 1959, Nature 183:1056-1057) that some strains of bacteria are sensitive to low levels of mitomycin. Therefore, to compare the sensitivity of a specific bacterial strain with and without the *sulA*::p450 OR expression plasmid the following experiment was performed. If the construct is functional, the presence of the p450 OR gene is expected to result in increased activation of mitomycin resulting in decreased growth of the bacteria. The *sulA*::p450 OR expression plasmid used in this experiment is similar to pSP-SAD4-5 (Section 13.3) except that it is in a pBluescript (pBS) backbone and has the β-galactosidase transcription unit. This construct is also diagramed in Figure 4-D. The pBS plasmid, with and without the expression construct, was transfected into *Escherichia coli* DH5α by electroporation and clones containing the correct plasmids were obtained and confirmed by plasmid isolation and DNA restriction analysis. For each of the two plasmid-bearing strains, a fresh, 4 hr (late log) culture was diluted 1:100 into LB with 100 μg/ml ampicillin to select for the presence of the plasmid and grown at 37°C at 250 rpms. Mitomycin C was added to the cultures in amounts of 0.0, 0.1 and 0.5 μg per ml.

Optical density was determined at 600 nm using a Perkin Elmer double beam spectrophotometer at 2 and 18 hour time points. The results are presented in Table 14(A).

TABLE 14A

GROWTH OF BACTERIAL CULTURES IN THE PRESENCE OF MITOMYCIN C

	OD_{600} t= 2 hours post drug Amount of Mitomycin C (μg/ml) added		
Plasmid	0	0.1	0.5
pBS	0.052	0.050	0.053
sulA::p450 OR	0.037	0.030	0.024

	OD_{600} t= 18 hours post drug Mitomycin C (μg/ml) added		
Plasmid	0	0.1	0.5
pBS	2.33	2.23	1.93
sulA::p450 OR	2.26	0.34	0.071

Comparison of the growth of the *E. coli* strain DH5α containing pBS and *sulA*::p450 OR in the absence of drug at the 2 and 18 hour time points shows that the presence of the construct partially inhibits the rate of growth but does not inhibit attaining a high final OD at 18 hours. These data also show that bacteria carrying the pBS backbone plasmid alone are only partially inhibited at the higher mitomycin concentration. However, those carrying the *sulA*::p450 construct show significant inhibition at both early and late time points at both mitomycin concentrations. These data indicate a strong dose response to mitomycin conferred by the presence of the *sulA*::p450 construct.

13.5. EXPRESSION OF CYTOSINE DEAMINASE IN *SALMONELLA TYPHIMURIUM*

E. coli cytosine deaminase (CD) has been shown to be an effective prodrug-converting enzyme useful for gene therapy (Hirschowitz et al., 1995, Human Gene Therapy 6:1055-1063; Huber et al., 1993, Cancer Res. 53:4619-4626; Huber et al., 1994, Proc. Natl. Acad. Sci. USA 91:8302-8306; Moolten,

1994, Cancer Gene Ther. 1:279-287; Mullen et al., 1992, Proc. Natl. Acad. Sci. USA 89:33-37; Mullen et al., 1994, Cancer Res. 54:1503-1506; Trihn et al., 1995, Cancer Res. 55:4808-4812). CD functions by converting the non-toxic 5-fluorocytosine (5-FC) to the toxic compound 5-fluorouracil (5-FU). *Salmonella* possess an endogenous CD, however, its expression is catabolite repressed (West and O'Donovan, 1982, J. Bacteriol. 149:1171-1174). A CD expression vector using the constitutively active β-lactamase promoter to ensure expression of CD within tumors was cloned as described below.

Cloning and expression of CD

PCR primers based on the complete sequence for *E. coli* cytosine deaminase (Huber et al., 1993, Cancer Res. 53:4619-4629) were forward: 5'-GATCATGCATGTGGAGGCTAACAGT-3' (SEQ ID NO:9) and reverse: 5'-CTAGATGCATCAGACAGCCGCTGCGAAGGC-3' (SEQ ID NO:10), corresponding to the published sequence, with the added sequence GATCATGCAT (portion of SEQ ID NO:9) or CTAGATGCAT (portion of SEQ ID NO:10) which is a *Nsi*I site and spacer at the 5' end of each primer. Each 25 μl reaction mixture contained 50 ng DNA template, 10 pmoles of each primer, 100 mM deoxynucleotide triphosphates, 1.5 mM Mg and 0.5 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Amplification was performed by 35 cycles of 94°C for 1 minute; 50°C for 15 seconds; 55°C for 1 minute; and 72°C for 2 minutes. The amplified DNA was purified on an agarose gel and the band of correct size was cloned into 1) pBluescript II KS+ and sequenced with T3 and T7 primers to confirm the correct DNA had been cloned and 2) p279 cut with PstI which provides the β-lactamase signal sequence and the constitutive β-lactamase promoter. This second construct was designated pCD-Sec1 and is diagramed in Figure 4-E. Transformants were screened using a [α-^{32}P]dCTP-labeled oligonucleotide probe. Positive clones were further screened by immunoblot using anti-CD antibodies described below.

Primary Antibodies to Cytosine Deaminase

CD was subcloned from pBluescript into pGEX I and expressed using IPTG. The expressed protein was found to be

insoluble and present in inclusion bodies. CD-glutathione-S-transferase (GST) fusion protein was purified from inclusion bodies by washing in 0.1% w/v Triton X-100, repelleted and resuspended in SDS-PAGE sample buffer. The material was separated on a 3 mm preparative 10% polyacrylamide gel and excised after visualization with 3M potassium acetate at 4°C. The purifed bands were homogenized and injected i.p. into DBA2J mice with Freund's complete (day 0) and incomplete (day 14) adjuvant. After 6 weeks the mice were bled and the ability of the serum antibodies to bind to cloned CD confirmed by Immunoblot.

SDS-PAGE and Immunoblot

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on bacterial lysates according to Weber and Osbom, 1975, Proteins and sodium dodecyl sulfate: Molecular mass determination on polyacrylamide gels and related procedures. In:H. Neurath and R. Hill (eds) The Proteins, Third Ed., Vol. I, Academic Press, New York, pp. 179-223. Immunoblots were performed according to Towbin et al., 1979, Proc. Natl. Acad. Sci. USA 76:4350-4354. Primary anti-CD antibodies described above were generally used at a 1:500 dilution. Secondary anti-mouse antibodies were alkaline phosphatase-conjugates (Promega, Madison, WI) used at a 1:7,500 dilution, followed by nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl phosphate (BCIP) colorimetric detection (Promega, Madison, WI).

CD Enzyme Assay

Bacterial lysates were prepared by pelleting 50 ml of overnight bacterial culture at 3000x g for 10 minutes and resuspending them in 2.5 ml of PBS. The cells were sonicated and the debris removed by pelleting in a microfuge at 12,000x g for 10 minutes. The enzyme assay performed was modified from Mullen et al., 1992, Proc. Natl. Acad. Sci. USA:89:33-37. 10 μl of cell extract was incubated with 1 μl [H^3]-5FC (1 μCI/μl) a 37°C. 1 μl was spotted on a Kodak 13254 microcrystalline nitrocellulose TLC plate, Eastman Kodak, Rochester New York, and separated using 95:5 Butanol:water

with unlabeled 5FC and 5FU markers. The plates were cut based upon separation of the marker lanes and quantified using a liquid scintillation counter.

Salmonella transformation

Transformation of *Salmonella* strains was performed by electroporation as described by O'Callaghan and Charbit, 1990, Mol. Gen. Genet. 223:156-158. Plasmids transfected into *Salmonella* were p279 and pCD-Sec1. *Salmonella typhimurium* strains transfected were strains YS721, YS7211, YS7212 and YS7213 which are described *infra* in Section 18. *E. coli* strains transfected were strains DH5α and KL498 (Δ*cod*).

Biodistribution of Salmonella carrying the CD expression construct

Salmonella typhimurium clone YS7212 carrying the CD expression construct pCD-Sec1 was grown in LB media to an OD_{600} of 0.8. An aliquot of 1.0×10^6 bacteria were inoculated i.p. into C57/B6 mice which had been implanted with 2×10^5 B16 melanoma cells 16 days prior to the bacterial infection. At two days post bacterial infection, mice were sacrificed and tumors and livers assayed for the presence of the bacteria by homogenization and plating of serial dilutions.

The expressed protein product of pCD-Sec1 bound to the anti-CD anti-serum by immunoblot analysis. When this clone was transferred to the *E. coli* strain KL498 which lacks CD, it was found to confer a high degree of enzyme expression as determined by the conversion of 5FC to 5FU, as shown in Figure 4-F. Figure 4-F also demonstrates that the cloned CD-expression plasmid gives higher levels of conversion than the *E. coli* strain MG1655 which expresses the wild type haploid *cod* gene which encodes for endogenous CD.

14. EXAMPLE: PROLIFERATION OF *SALMONELLA TYPHIMURIUM* CLONE #72^{5-3-2} IN MELANOMA TUMORS IN MICE

In a similar set of experiments as in Section 10.2, DBA/2J mice (approximately 10 weeks) were inoculated (s.c.) with $3x10^5$ Cloudman S91 melanoma/macrophage hybrid #48 cells

in each of 4 sites over the right and left shoulders and flanks. Tumors were palpable 10-12 days post inoculation of tumor cells from tissue culture into mice. After two weeks post-inoculation of tumor cells (s.c.), tumor-bearing mice were additionally inoculated (i.p.) with $2x10^5$ c.f.u. of *S. typhimurium* clone 72 containing the *HSV TK* gene with the β-lactamase signal sequence which is designated 72^{5-3-2}. After 2 and 10 days of bacterial infection without antibiotic treatment, representative tumor-bearing animals were sacrificed and their tumors and livers were homogenized and quantitated for c.f.u. of *Salmonella* per gram of tissue. In addition, individual clones of bacteria were isolated from the liver and tumor homogenates 10 days post-infection and tested for the genetic markers xyl^{neg} (inability to metabolize xylose, characteristic of clone #72) and tet^{res} (resistance to the antibiotic tetracycline). The genotype of the inoculated *Salmonella typhimurium* clone $\#72^{5-3-2}$ was xyl^{neg} and tet^{res}. The tet^{res} clones of *Salmonella* were assumed to carry the HSV TK gene, since the HSV TK gene was carried on a plasmid that carried the tet^{res} marker.

The results are presented in Tables 15 and 16. After 2 days of infection, the tumors contained an average of 1.5×10^9 *Salmonella*/g tumor and 2.0×10^5 *Salmonella*/g liver, with an average ratio of tumor:liver of about 7,500:1.

TABLE 15

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 2 DAYS FOLLOWING INOCULATION (I.P.) INTO CLOUDMAN S91 MELANOMA-BEARING DBA/2J MICE

Tissue	*Salmonella*/g Tissue	Tumor/Liver
Liver (n = 2)	2.0×10^5	---
Tumor (n = 4)	$1.5 \pm 0.9 \times 10^9$	7,500:1

After 10 days of infection (Table 16), the tumors contained an average of 2.9×10^9 *Salmonella*/g tumor and 2.7×10^5 *Salmonella*/g liver, a ratio of 11,000:1 (tumor:liver), similar to the distribution of bacteria seen 1-2 days post-infection. These results demonstrate that once inoculated (i.p.) into tumor-bearing mice, *Salmonella* enter the circulatory system, infect the tumor cells, proliferate within the tumors, and exist there in a compartmentalized fashion.

TABLE 16

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 10 DAYS FOLLOWING INOCULATION (I.P.) INTO CLOUDMAN S91 MELANOMA-BEARING DBA/2J MICE

Tissue	*Salmonella*/gm Tissue	Tumor/Liver	xylose	tetracycline
Liver	2.7×10^5	---	neg	9/10 res
Tumor #1	4.2×10^9	16,000:1	neg	3/7 res
#2	3.1×10^9	12,000:1	neg	7/8 res
#3	1.3×10^9	4,800:1	neg	8/8 res
Average	2.9×10^9	11,000:1		

The results further demonstrate that 10 days post-infection, all of the bacterial clones examined were xyl^{neg}, proving their genetic relationship to the inoculated clone

#72^{5-3-2}, and that 27/33 clones remained *tet*^{res}, demonstrating high degree of retention (82%) of the *HSV* TK containing plasmid within the host bacteria. In experiments not shown here, the same plasmid was found to be 100% retained after 42 hours of infection in tumor-bearing mice.

In a continuation of the above experiments summarized in Tables 14, 15 and 16, the *Salmonella* infections in melanoma-bearing mice were continued for a total of 4 weeks. To alleviate the symptoms of *Salmonella* poisoning (shaking, matted hair) the animals were placed on antibiotics for the final two weeks. Such antibiotic treatments consisted of the inclusion in the mouse drinking water of SULFATRIM™ Pediatric Suspension (Schein Pharmaceutical, Inc.; sulfamethoxazole 40 mg/ml, trimethoprim 8 mg/ml) at a final concentration of 15 ml SULFATRIM™/500 ml drinking water. At termination of the experiment, the surviving mice were sacrificed by euthanasia, and the tumors and livers were removed. Portions of the tissues (1-2 mm^3) were fixed in formalin and stained for histological examination. The remaining portions were weighed, homogenized in 5 ml LB broth/g tissue, and the number of *Salmonella* were quantitated on LB agar plates. Results are presented in Table 17.

TABLE 17

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 4 WEEKS FOLLOWING INOCULATION (I.P.) INTO MELANOMA-BEARING DBA/2J MICE

Tissue	*Salmonella*/g tissue	Tumor/Liver
Liver	5.1×10^7	---
Tumor		
#1	2.2×10^9	43:1
#2	9.4×10^8	18:1
#3	2.3×10^9	45:1
#4	2.0×10^8	6:1
Average	$1.4 \pm 1 \times 10^9$	28:1

The excised melanoma tumors averaged less than 1 gram in weight compared to 5-10 gm tumors in control animals at death (data not shown). It was found that these tumors contained an average of 1.4×10^9 *Salmonella*/g tumor, similar to the number of tumor-infecting bacteria seen at 1, 2, and 10 days post-inoculation. However, the number of *Salmonella* in the liver increased during the 4 week infection, so that the average ratio of bacteria in tumor over liver was reduced to 28:1 compared to the ratios obtained with infection periods of up to 10 days as seen in Tables 14, 15 and 16.

15. EXAMPLE: MICROSCOPIC DETECTION OF *SALMONELLA TYPHIMURIUM* IN MELANOMAS IN VIVO

15.1. DETECTION OF *SALMONELLA TYPHIMURIUM* WITHIN CLOUDMAN S91 MELANOMAS GROWING IN DBA/2J MICE

In order to study the histopathology of *Salmonella* infection in the tumor-bearing mice, representative melanoma tumors were removed from euthanized mice with or without *Salmonella* infection. Portions of the tissues (1-2 mm^3) were fixed in formalin, embedded and sectioned, and the sections stained with either hematoxylin and eosin, or tissue gram stain for histological examination. Results of these studies are shown in Figures 5A-B.

Figures 5A-B are photomicrographs of histological sections from a Cloudman S91 melanoma/macrophage hybrid #48 melanoma growing subcutaneously in a DBA/2J mouse. The tumor was excised from a mouse that had been inoculated 2 days earlier with 3×10^5 c.f.u. *Salmonella typhimurium* super-infective clone 72 carrying the HSV TK gene, 72^{5-3-2}. A portion of the tumor was weighed, immersed in LB at 5ml/g tumor, homogenized with a ground glass homogenizer, and the tumor homogenate was plated onto LB-Agar culture plates at various dilutions in order to quantitate the amount of *Salmonella typhimurium* in the tumor. Quantitation of the bacteria revealed that the tumor contained 1.4×10^9 *Salmonella*/g. Figure 5A. A section stained with hematoxyn

and eosin shows a cross-section of the tumor with an area of necrosis, denoted by the arrow. Figure 5B. A section stained with tissue gram stain shows gram negative bacteria in an area of necrosis area of the tumor. When viewed with the light microscope, the bacteria stain pink/purple against a yellow background. *Salmonella*-infected necrotic areas were surrounded with dead tumor cells that did not stain with tissue gram stain but which could be detected through melanin-containing melanosomes (see Figure 6). These results show that the necrotic areas of solid tumors are accessible to *Salmonella* when the bacteria are introduced into a tumor-bearing host via the circulatory system.

In an additional set of analyses, sections of Cloudman S91 melanoma/macrophage hybrid #48 melanoma tumors growing in a *Salmonella*-infected mouse were examined with the electron microscope. To initiate the experiment, a mouse was inoculated s.c. with 8×10^5 tumor cells. A palpable tumor mass was detected 11 days later, at which time the mouse was inoculated i.p. with 3.6×10^6 c.f.u. of *S. typhimurium* super-infective clone #72. Forty-two hours post-inoculation, the mouse was sacrificed by metofane anesthesia. The tumor was excised using aseptic techniques. Quantitation of the bacteria within the tumor revealed that the tumor contained approximately 7.5×10^9 *S. typhimurium*/g upon excision at 42 hours. In contrast, the concentration of *S. typhimurium* in the liver from the same mouse was approximately 2.0×10^7/g, a ratio of bacteria in tumor to liver of approximately 400:1.

A second portion of the tumor was cut into 1-2 mm^3 pieces and fixed in 1/2 strength Karnovsky's fixative for 6 hours at 4°C, followed by washing in cacodylate buffer overnight. The tumor tissue was post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide in cacodylate buffer for 2 hours and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate. They were viewed with a Zeiss 109 electron microscope.

Shown in Figure 6 is an electron micrograph of a field within a melanoma tumor that includes two separate *S.*

typhimurium along with numerous melanosomes, which are specialized subcellular organelles present in the cytoplasm of melanoma cells. The presence of bacteria along with the melanosomes provides proof that the *S. typhimurium* entered the cytoplasm of the melanoma cell via the bloodstream of the mouse. The *S. typhimurium* in the electron micrograph appear identical to those shown previously in intestinal epithelial cells following an experimental infection of the mouse, Takeuchi, 1967, Am. J. Pathol. 50:109-1361.

In summary, i) examination with the light microscope revealed that *Salmonella typhimurium* exists in the necrotic areas of Cloudman S91 melanomas growing in infected DBA/2J mice; and ii) examination with the electron microscope revealed that *Salmonella typhimurium* also exists within the cytoplasm of melanoma tumor cells.

15.2. DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* WITHIN MOUSE B16F10 MELANOMA TUMORS GROWN IN C57BL/6J MICE

C57BL/6J mice (11-13 weeks old) were inoculated s.c. in two sites (shoulder and flank), with 3.5 x 10^5 B16F10 mouse melanoma cells per site. After the appearance of palpable tumors (approximately 2 weeks) the animals were further inoculated i.p. with about 10^5 bacteria of the following three strains: i) wild type *Escherichia coli* K-12 strain #CSH 101; ii) *Salmonella typhimurium* strain 14028; and iii) mutant *Salmonella typhimurium* super-infective clone 72 carrying the *HSV* thymidine kinase gene, 72^{5-3-2}. After about 2 days of infection, mice were euthanized by anesthesia with metofane. Tumors and livers were removed aseptically, rinsed with sterile 0.9% NaCl, weighed, and homogenized in LB broth at a ration of 5:1 (vol. broth:wt. tumor). Prior to homogenization, 1-2 mm^3 pieces of tissue were removed from representative tumors, fixed with 1/2 strength Karnovsky's fixative, and processed for analysis with the electron microscope. Bacteria in the homogenates were quantitated by

plating onto LB plates, incubating overnight at 37°C, and counting bacterial colonies.

Results were as follows: i) wild type *E. coli* were found in relatively low numbers in both the tumor and liver of the inoculated animals at concentrations averaging $<10^3$/g tumor and $<10^2$/g liver. ii) wild type *S. typhimurium* were found in significantly higher numbers than *E. coli* in both tumor and liver, with infecting bacteria ranging from 2×10^7 to 6×10^8 c.f.u./g tumor, and 4×10^6 c.f.u./g liver. One of the two C57BL/6J mice inoculated with the wild type *S. typhimurium* strain died, possibly from septic shock. iii) *S. typhimurium* super-infective clone 72 were also found in significantly higher numbers than *E. coli* in both tumor and liver, further, the number of clone 72 *S. typhimurium*/g liver was significantly lower than the number of wild type *S. typhimurium*/g liver. The results are detailed below in Table 18.

TABLE 18

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI* 2 DAYS FOLLOWING INOCULATION (I.P.) INTO C57B6 MICE BEARING B16F10 TUMORS

Bacterial Strain	Mouse	Tissue	Bacteria/ gm Tissue	Tumor/ Liver
E. coli K-12 (CSH #101)	A	Liver	355	—
		Tumor #1	1200	4:1
		Tumor #2	50	1:7
	A'	Liver	100	—
		Tumor #1	50	1:7
S. typhimurium (14028 wild type)	B	Liver	4.3×10^6	—
		Tumor #1	2.3×10^7	5:1
		Tumor #2	6.0×10^8	136:1
	B'	(dead)	—	—

S. typhimurium (clone # 72^{5-3-2})	C	Liver	2.0×10^4	___
		Tumor #1	1.0×10^8	5,000:1
		Tumor #2	1.2×10^5	6:1
	C'	Liver	8.5×10^4	___
		Tumor #1 (2.5g)	9.3×10^8	11,000:1

In summary, *Salmonella typhimurium* displays natural capabilities over *Escherichia coli* in its ability to infect and proliferate within B16F10 melanoma tumors. Furthermore, super-infective clone 72^{5-3-2} displays superior qualities to its wild type parental strain 14028 in its reduced infection of liver in C57BL/6J mice, *i.e.*, the wild-type strain 14028 showed greater infectivity toward liver than did clone 72^{5-3-2}. The higher infectivity of liver by the wild-type *Salmonella* was consistent with the observed greater lethality of the wild type stain toward DBA/2J mice and the greater infectivity of liver in DBA/2J mice than that produced by clone 72 as seen in Table 11B. Together, the results in Tables 11B and 18 provide the first evidence that selection for strains of bacteria or other parasites with enhanced tumor specificity *in vitro* can yield mutant strains with attenuated host toxicity *in vivo*.

15.3. MICROSCOPIC DETECTION OF *SALMONELLA TYPHIMURIUM* WITHIN B16F10 MELANOMAS GROWING IN C57BL/6J MICE

Representative B16F10 melanoma tumors were removed from euthanized mice with or without *Salmonella* infection. Portions of the tissues (1-2 mm^3) were fixed in formalin, embedded and sectioned, and the sections stained with either hematoxylin and eosin, or tissue gram stain for histological examination. Results of these studies are shown in Figures 7A-B. Figures 7A and 7B are light micrographs of histological sections from a B16F10 melanoma growing

subcutaneously in a C57BL/6J mouse. The tumor was excised from a mouse that had been inoculated 2 days earlier with 2 x 10^5 c.f.u. *Salmonella typhimurium* super-infective clone, 72^{5-3-2} carrying the *HSV* TK gene. Quantitation of the bacteria within the tumor revealed that the tumor contained approximately 9 x 10^8 c.f.u. *S. typhimurium*/g upon excision 2 days post-infection with bacteria. In contrast, the concentration of *S. typhimurium* in the liver from the same mouse was approximately 2.0 x 10^5/g, a ratio of bacteria in tumor to liver of approximately 400:1. Figure 7: A section stained with tissue gram stain shows gram negative bacteria in an area of necrosis within the tumor. The infected necrotic area is surrounded by dead melanoma cells that do not stain with the tissue gram stain but which appear brown in color due to the presence of melanized melanosomes. When viewed with the light microscope, the bacteria stain pink/purple against a yellow background. The results show that necrotic areas of B16 melanoma tumors are accessible to *Salmonella* when the bacteria are introduced into a tumor--bearing host via the circulatory system.

A second portion of the above-described tumor was cut into 1 mm^3 pieces and fixed in 1/2 strength Karnovsky's fixative for 6 hours at 4°C, followed by washing in cacodylate buffer overnight. The tumor tissue was post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide in cacodylate buffer for 2 hours, and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate. They were viewed with a Zeiss 109 electron microscope as depicted in Figure 8.

The electron micrograph in Figure 8 shows numerous *Salmonella typhimurium* in extracellular spaces, denoted by arrows, in an area of necrosis. A single bacterium is also seen within the cytoplasm of a dying melanoma cell. The cytoplasm of the dying melanoma cell also contains numerous black melanosomes, characteristic of the B16F10 melanoma.

The *S. typhimurium* in the electron micrograph appear identical to those shown previously in intestinal

epithelial cells following an experimental infection of the mouse, Takeuchi, 1967, Am. J. Pathol. 50:109-136.

In summary, i) examination with the light microscope revealed that *Salmonella typhimurium* exist abundantly in the necrotic areas of B16F10 melanomas growing in infected B16F10 mice; and ii) examination with the electron microscope revealed that *Salmonella typhimurium* also exist within the cytoplasm of tumor cells. *Salmonella* were also observed in tumor-associated neutrophils.

16. EXAMPLE: USE OF SUPER-INFECTIVE TUMOR-SPECIFIC GENE-DELIVERING *SALMONELLA TYPHIMURIUM* FOR TREATMENT OF MICE BEARING MELANOMA TUMORS

16.1. TREATMENT OF CLOUDMAN 591 MELANOMA

Salmonella typhimurium super-infective mutant 72^{5-3-2}, constitutively expressing the *Herpes simplex* virus thymidine kinase gene with the β-lactamase signal sequence, was used for gene therapy of melanoma in mice (see Figure 4-C). DBA/2J mice (approximately 10 weeks) were inoculated (s.c.) with 3 x 10^5 Cloudman S91 melanoma/macrophage hybrid cells in each of 4 sites over the right and left shoulders and flanks. Tumors were palpable 10-12 days post-inoculation of tumor cells.

After two weeks post-inoculation of tumor cells, tumor-bearing mice were further inoculated (i.p.) with 2 x 10^5 c.f.u. of *S. typhimurium* clone 72 containing the *HSV* thymidine kinase gene with the β-lactamase secretory signal sequence which is designated 72^{5-3-2}. Twelve hours after inoculation of the bacteria, some of the mice were further inoculated (i.p.) with 2.5 mg ganciclovir sodium (Cytovene, Syntex Laboratories, Palo Alto, Calf.) in isotonic saline. These same mice received this dosage of ganciclovir four times over a 3 day period. Control tumor-bearing mice also received ganciclovir but no bacteria. Another set of tumor-bearing mice was inoculated with bacteria, but received no ganciclovir. At various times appropriate groups of mice, treated as above, were also given the antibiotic Sulfatrim™

Pediatric Suspension (Schein Pharmaceutical, Inc.; sulfamethoxazole 40 mg/ml, trimethoprim 8 mg/ml) at a concentration of 15 ml Sulfatrim™/500 ml drinking water.

Results were as follows:

1) Control melanoma tumor-bearing mice, receiving ganciclovir and antibiotic treatment (Sulfatrim™ in drinking water) but no bacteria, developed rapidly growing tumors that initially doubled in size every 3 to 4 days, determined by caliper measurements as shown in Figure 9. These animals exhibited little or no side-effects from the ganciclovir treatment, confirming previous reports on the minimal toxicity of the ganciclovir pro-drug in mice in the absence of a suitable thymidine kinase converting enzyme (Bonnekoh et al, 1995, J. Invest. Dermatol. 104:313-317). By 30 days post-inoculation with tumor cells, all mice in this group had formed massive subcutaneous tumors (5-10 gm) and had died from melanoma.

2) One group of tumor-bearing mice received bacteria for a total of 10 days without administration of antibiotics, and received no ganciclovir. These animals had tumors that were significantly reduced in size from the tumors in control mice (Figure 10). The effect of *Salmonella* alone on reducing tumor size became evident several days after the effect of *Salmonella* plus ganciclovir on tumors had been observed as described below. However, all the animals in the "*Salmonella* alone" group developed symptoms of *Salmonella* infection (shaking, matted hair) and 50% of these animals succumbed between 5-10 days post-infection. The remaining animals were treated with the antibiotic Sulfatrim™ (Schein Pharmaceutical, Oral Suspension) at a concentration of 15 ml/500 ml drinking water. This treatment reduced the clinical symptoms of Salmonella infection in the mouse population within 24-48 hours. The surviving animals from this protocol had significantly smaller tumors than control animals and remained alive past the 30 day period, when all of the control animals had died from melanoma.

3) Another group of tumor-bearing mice received ganciclovir plus bacteria during a 4-day treatment period. About 50% of the animals succumbed within 1-2 days of this treatment, apparently from the conversion of ganciclovir to its toxic, phosphorylated form by the *HSV* TK expressed by the *Salmonella* clone 72^{5-3-2} within the body of the mouse. At this time ganciclovir treatment was discontinued and the surviving animals were placed on Sulfatrim™ antibiotic to control the *Salmonella* infection. The total time of exposure to *Salmonella* without antibiotic was 4 days. The survivors from this protocol had significantly smaller tumors than control animals and remained alive past the 30 day period when all the control animals had died from melanoma (Figure 11).

In a further set of experiments, tumor progression was measured with calipers in various treated and untreated tumor-bearing mice. Groups of mice bearing Cloudman S91 melanoma/macrophage hybrid #48 melanoma tumors as described above were inoculated (i.p.) with 3 x 10^5c.f.u. *Salmonella typhimurium* super-infective clone 72 carrying the *Herpes simplex* virus thymidine kinase gene, 72^{5-3-2}. Twenty-four hours after inoculation with bacteria, the mice were further inoculated with ganciclovir at doses of 2.0 mg, with a total of 6 inoculations over a 5 day period. The mice were then subjected to antibiotic treatment with a combination of 15 ml/500 ml Sulfatrim™ and 20 μg/ml Baytril™ (Miles) in their drinking water. Baytril™ or enrofloxacin is 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid. Tumor growth was assessed with periodic caliper measurements of tumor length, width, and height, and computed as tumor volume in mm^3 by techniques known to the science of tumor biology. Results were plotted on a semi-logarithmic scale and generation times, the time in hours for one doubling in volume, were calculated. The following formula was used:

Generation time = $0.69\ (t)/\ln\ (T_1/T_2)$, where t equals the time in hours between the initial tumor volume (T_1)

and the final tumor volume (T_2) over the linear portion of the curve.

The results are shown in Figure 9 and Table 19. Mean doubling times of tumors in untreated control mice and mice treated with ganciclovir but no *Salmonella* infection were similar, being 83 and 94 hours respectively. Tumors in mice treated with *Salmonella* for 5 days but no ganciclovir doubled at a mean rate of 125 hours. Tumors in mice treated with *Salmonella* for 5 days as well as ganciclovir showed no growth over the 10 day measurement period, and in some cases regressed with the treatment.

TABLE 19

EFFECTS OF *HSV TK*-CONTAINING *SALMONELLA TYPHIMURIUM* ON THE GROWTH OF CLOUDMAN S91 MELANOMAS IN DBA/2J MICE ± TREATMENT WITH GANCICLOVIR

Treatment	Mean Tumor Doubling Time (hrs)
none	83
ganciclovir	94
S. typhimurium	125
S. typhimurium + ganciclovir	no growth

In summary: a) Control tumor-bearing animals receiving ganciclovir and antibiotic treatment, but no *Salmonella*, succumbed from massive tumors within 30 days of inoculation of tumor cells; b) Animals receiving *Salmonella* alone followed by antibiotic treatment showed reduced tumor growth rate and prolonged survival over control animals; c) Animals receiving a combination of ganciclovir and *Salmonella* followed by antibiotic treatment showed little or no tumor growth compared to control animals, and prolonged survival over control animals. The results indicate that *Salmonella typhimurium* expressing the *Herpes simplex* virus thymidine kinase gene was able to convert ganciclovir to its

phosphorylated form within the melanoma tumors, thus reducing tumor size and prolonging survival of the mice.

16.2. **TREATMENT OF B16F10 MELANOMA**

C57B6 mice were inoculated s.c., left shoulder region, with 5 x 10^5 B16F10 melanoma cells from culture. At 8 days post-tumor implantation some of the mice were further inoculated i.p. with 2 x 10^6 c.f.u. attenuated *Salmonella typhimurium* strains YS721, YS7211, YS7212 or YS7213 (see Section 18, *infra*) each carrying the HSV TK gene. At 11 days post-tumor implantation, GCV (ganciclovir sodium, CYTOVENE™, Syntex Laboratories, Palo Alto, CA.) was inoculated i.p. into groups of mice (n=5 or n=10) under the following treatment protocols: a) total dose = 7.5 mg/mouse (2.5 mg day 11, 1.25 mg day 12; 2.5 mg day 18, 1.25 mg day 19); b) total dose = 5.0 mg/mouse (2.5 mg day 11, 2.5 mg day 12); c) total dose = 3.75 mg/mouse (2.5 mg day 11, 1.25 mg day 12); d) total dose = 2.5 mg/mouse (1.25 mg day 11, 1.25 mg day 12); e) total dose = 1.25 mg/mouse (1.25 mg day 11). At 18 days post-tumor implantation (10 days post bacterial inoculation) all animals were given enrofloxacin antibiotic, 0.2 mg/ml, in their drinking water (BAYTRIL™) and maintained with this antibiotic supplement for 2 weeks. Tumor growth was assessed by caliper measurements and computed as volume in mm^3. Animals were euthanized and listed as dead when the sum of their tumor measurements, length + width + height, reached 60 mm, or when they became moribund (listless, cessation of drinking).

The results obtained are illustrated in Figure 11C-H in Table 19(A).

TABLE 19(A)

SURVIVAL OF C57B6 MICE INOCULATED WITH *SALMONELLA TYPHIMURIUM* ± HSV TK GENE: EFFECTS OF GANCICLOVIR

Strain	(n=)	GCV	Time Days ± S.D.	Treated/Control T/C
Control	(10)	-0-	25±0	1.0
	(10)	3.75mg	27±1	1.1
YS7212	(10)	-0-	42±2	1.7
YS7212	(10)	3.75mg	33±4	1.3
YS7212/p5-3	(10)	-0-	45±3	1.8
YS7212/p5-3	(10)	3.75mg	40±4	1.6
YS7213	(10)	-0-	34±2	1.3
YS7213	(10)	3.75mg	35±2	1.4
YS7213/p5-3	(10)	-0-	29±2	1.2
YS7213/p5-3	(10)	3.75mg	33±2	1.3
YS7211	(10)	-0-	40±4	1.6
YS7211	(10)	3.75mg	35±4	1.4
YS7211/p5-3	(10)	-0-	34±2	1.4
YS7211/p5-3	(5)	1.25mg	30±4	1.2
	(5)	2.50mg	37±4	1.5
	(5)	3.75mg	38±4	1.5
	(5)	5.0mg	42±6	1.7
	(5)	7.5mg	39±6	1.6

*Time of death post tumor cell inoculation.

The results from the various treatment protocols for the B16F10 melanoma-bearing mice were as follows:

1) Effects of GCV on tumor-bearing animals with no bacterial inoculation

Mice receiving melanoma cells but no bacteria were treated with GCV on days 11-12 post inoculation with tumor cells at doses from 3.75 mg-10 mg/mouse, depending on the experiment. In all trials, mice treated with GCV but no bacteria showed a small reduction in tumor volume that was noticeable within 5 days of GCV treatment and which persisted through the duration of the experiment, as shown in Figure

11C-E. GCV also elicited small but reproducible increases in survival time compared to that of non-treated control animals, as outlined in Table 19(A). These effects of GCV in the absence of bacterial treatment were not dependent upon dosage over the range studied.

These results demonstrate that the B16F10 cells employed in the experiment might have had the capacity to convert GCV to its toxic, phosphorylated form. Consistent with such a notion, it was found that proliferation of the B16F10 melanoma cells in culture was significantly suppressed when GCV was supplemented to the culture medium at 25 μg/ml, but not at 10 μg/ml as shown in Figure 11-F. Similar effects of GCV on DBA/2J mice bearing Cloudman S91 melanoma x macrophage hybrid 48, but not inoculated with bacteria, are reported in the Table 19.

2) Effects of GCV on tumor-bearing animals treated with bacteria not containing an HSV TK-plasmid

When tumor-bearing mice were inoculated with *Salmonella* strains YS7211, YS7212 and YS7213, none of which contained the *HSV* TK gene, and then treated with GVC, GCV-mediated suppression of tumor growth was evident. Tumor suppression achieved with GCV was significantly greater than that seen with the bacteria alone, even when the suppressive effect of GCV on B16F10 tumors in control animals was taken into account. This indicated that *Salmonella typhimurium* could convert GCV to its phosphorylated, toxic form without the *HSV* TK gene, perhaps through endogenous phosphotransferase enzymes (Littler, et al., 1992, Nature 358:160-162; Sullivan et al., 1992, Nature 358:362-364). Consistent with this notion was the finding that in addition to suppressing tumor growth, some combinations of bacteria and GCV treatment were highly toxic, shortening survival times of the animals, shown in Table 19(A). Toxicity might have resulted from production of phosphorylated GCV by those bacteria located in normal tissues such as liver or bone marrow.

3) Effects of GCV on tumor-bearing animals treated with bacteria containing an HSV TK-plasmid

Tumor-bearing mice inoculated with *HSV* TK plasmid-containing *Salmonella* clone YS7211 (YS7211/p5-3) showed suppression of tumor growth and prolonged survival even in the absence of GCV treatment as shown in Figure 11-G. Further, animals bearing both tumors and YS7211/p5-3 and additionally treated with 3.75mg GCV showed significant suppression of tumor growth above that seen in the absence of GCV. Using YS7211/p5-3 as a vector, GCV-mediated tumor suppression was evident in a dose-responsive manner when measured 28 days post implantation of tumors as shown in Figure 11-H. Tumor suppression correlated with increased average survival times for some categories of GCV-treated, tumor-bearing mice when compared to those inoculated with YS7211/p5-3 but not receiving GCV.

In summary:

1) In tumor-bearing animals not inoculated with *Salmonella*, GCV had a small suppressive effect on tumor growth that correlated with a small prolongation of survival.

2) Tumor-bearing animals inoculated with *Salmonella* not containing the *HSV* TK plasmid showed marked tumor suppression in response to GCV, above that seen in animals not treated with bacteria. In addition, some combinations of GCV and bacterial treatment were highly toxic to the animals, possibly through conversion of GCV to its toxic form by bacteria in extra-tumoral tissues such as liver or bone marrow.

3) Tumor-bearing animals inoculated with *Salmonella* containing the *HSV* TK plasmid also showed strong tumor suppression in response to GCV. It was not possible in these experiments to evaluate the relative contributions of *HSV* TK as compared to endogenous *Salmonella* enzymes in the phosphorylation of GCV. However, using as a vector clone YS7211 containing the *HSV* TK expression plasmid, GCV-mediated

tumor suppression and prolonged survival was demonstrated in a dose-dependent manner, see Figure 11-H.

17. EXAMPLE: LOCALIZATION OF *SALMONELLA TYPHIMURIUM* WITHIN HUMAN TUMORS GROWN IN *NU/NU* MICE

The following experiments demonstrate localization of *Salmonella* in human tumors in experimental animals.

17.1. LOCALIZATION OF *SALMONELLA* WITHIN HUMAN COLON TUMORS

NU/NU (BALB C) mice (9-10 weeks old) were inoculated s.c. in two areas (shoulder and flank), each with 1.5×10^7 HCT 116 human colon carcinoma cells. After the appearance of palpable, vascularized tumors (approximately 2 weeks) the animals were further inoculated i.p. with 3×10^5 *Salmonella typhimurium* super-infective clone 72^{5-3-2} carrying the *HSV* thymidine kinase gene. After 3.5 hours, 21 hours, and 72 hours of infection, mice were euthanized by anesthesia with metofane. Tumors and livers were removed aseptically, rinsed with sterile NaCl (0.9%), weighed, and homogenized with LB broth at a ration of 5:1 (vol. broth:wt. tumor). At 72 hours, prior to homogenization, pieces (1-2 mm^3) were removed from representative tumors, fixed with 1/2 strength Karnovsky's fixative, and processed for analysis with the electron microscope. Bacteria in the homogenates were quantitated by plating onto LB plates, incubating overnight at 37°C, and counting bacterial colonies.

Results were as follows: At 3.5 hours and 21 hours there were insignificant levels of bacteria in the tumors or livers, even when the homogenates were plated undiluted onto LB agar plates. However, after 3 days 3/6 animals displayed high levels of *Salmonella* in the colon tumors, with bacterial tumor:liver ratios ranging up to 36,000:1. Data for these animals are summarized below in Table 20.

TABLE 20

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 3 DAYS FOLLOWING INOCULATION (I.P.) INTO HUMAN COLON CARCINOMA-BEARING *NU/NU* MICE

Mouse A	*Salmonella*/ gm tissue	Tumor/ Liver
Liver	2.6×10^4	
Tumor	6.9×10^8	26,500:1
Mouse D		
Liver	1.6×10^6	
Tumor	3.1×10^9	2,000:1
Mouse E		
Liver	1.0×10^5	
Tumor	3.6×10^9	36,000:1

Shown in Figure 12-A an electron micrograph of a section from the HCT colon tumor excised from mouse A (Table 20) in which the number of *Salmonella* found to be 6.9×10^8/g tumor, and the tumor:liver ratio of infecting bacteria was 26,500:1. Shown in the micrograph are numerous *Salmonella typhimurium* within a vacuole in the cytoplasm of a neutrophil associated with the tumor. Some of the bacteria are undergoing division as denoted by the arrow. The neutrophil or polymorphonucleoleukocyte is characterized by its multi-lobed nucleus (n). *Salmonella* in tumor-associated neutrophils was also seen in infected B16F10 melanomas as described herein. The presence of bacteria in both colon and melanoma tumor-associated neutrophils following infection of tumor-bearing mice suggests that the *Salmonella* may have stimulated a host cellular immune response to the tumor cells. Enhancement of tumor immunity is thus another potential advantage in the use of parasites as tumor-specific therapeutic vectors.

17.2. LOCALIZATION OF *SALMONELLA* WITHIN VARIOUS HUMAN TUMORS

Nu/nu (BALB C) mice (9-12 weeks old) were inoculated s.c. in the left shoulder region with 1-1.5 x 10^7 cells of the human lung carcinoma A549, human colon carcinoma HCT 116, human renal carcinoma CRL 1611, or human hepatoma HTB 52 (American Type Culture Collection). When palpable tumors developed, the mice were inoculated further with 2-5 x 10^6 cfu *Salmonella typhimurium* clone 72 for animals bearing human lung, liver, and renal tumors, and clone 72^{5-3-2} for animals bearing human colon tumors. Clone 72^{5-3-2} carries the *HSV* thymidine kinase transcription unit. After 66-96 hours the animals were sacrificed, and the tumors and livers were removed and weighed. The tumor was homogenized in 5 vol LB broth/gram wet weight tissue. Homogenates were quantitated by serial dilution on LB agar plates for the number of bacteria. The results are presented in Table 20(A) and represent the average ± standard deviation for n=3-4 animals.

TABLE 20(A)

BIODISTRIBUTION OF *SALMONELLA TYPHIMURIUM* CLONE 72 IN *NU/NU* MICE BEARING HUMAN CARCINOMAS OF THE LUNG, COLON, KIDNEY, AND LIVER

	Salmonella/g tissue:			
Primary Tumor	Tumor	Liver	Tumor wt (mg)	Tumor: Liver
lung carcinoma	$3.2 \pm 1.4 \times 10^9$	$1.0 \pm 0.3 \times 10^7$	462 ± 186	320:1
colon carcinoma	$2.5 \pm 1.6 \times 10^9$	$5.8 \pm 8.9 \times 10^5$	428 ± 235	4300:1
hepatoma	$6.7 \pm 11 \times 10^8$	$5.7 \pm 9.0 \times 10^6$	103 ± 29	120:1
renal carcinoma	$1.4 \pm 1.8 \times 10^8$	$6.0 \pm 3.0 \times 10^5$	103 ± 99	230:1

As shown in Table 20-A, when inoculated i.p. into *nu/nu* mice, *Salmonella typhimurium* clone 72 was able to

target human carcinomas of the lung, colon, kidney, and liver, and proliferate within them, generally, but not always, reaching levels of 10^8-10^9/g tumor. In the BALB/c *nu/nu* mice used, the skin was hairless and translucent allowing it to be determined visually that all the tumors were vascularized. The ranges of wet weights of the *Salmonella*-infected tumors were lung carcinoma, 220-600 mg; colon carcinoma, 160-600 mg; hepatoma, 70-120 mg; and renal carcinoma, 40-250 mg.

Bacterial colonies were picked randomly from liver and tumor homogenates obtained from renal carcinoma- and hepatoma-bearing *nu/nu* mice 96 hrs post-inoculation of clone 72 and tested for phenotype by replicate plating. In all homogenates tested, 50/50 colonies were found to be Ade$^-$ and Xyl^{neg}, consistent with the clone 72 phenotype.

The results further support the notion that derivatives of *Salmonella typhimurium* are useful as therapeutic vectors for a broad range of solid tumors, independent of the tumor origin or size. In several studies *Salmonella* clone 72 and its derivatives targeted and amplified within highly vascularized tumors as small as 40-100 mg in the case of human tumors in *nu/nu* mice, as well tumors of 4-8 g with large necrotic areas in the case of B16F10 melanomas in C57B6 mice. The ability to target and amplify within small vascularized tumors presents a distinct advantage of *Salmonella typhimurium* as a therapeutic tumor vector.

17.3. LOCALIZATION BY ELECTRON MICROSCOPY OF *SALMONELLA TYPHIMURIUM* WITHIN HUMAN LUNG CARCINOMA A549

A mouse was inoculated s.c. in the left shoulder region with 5 x 10^6 A549 cells. After 6 weeks the tumor was palpable and the animal was inoculated i.p. with 3 x 10^6 *Salmonella typhimurium* clone 72, for 66 hours. The animal was sacrificed and a portion of the tumor was homogenized and found to contain 1.6 x 10^9 *Salmonella typhimurium*/g. The

central portion of the tumor was prepared for electron microscopy as follows: The portion of the tumor was cut into 1-2 mm^3 pieces and fixed in 1/2 strength Karnovsky's fixative for 6 hours at 4°C, followed by washing in cacodylate buffer overnight. The tumor tissue was post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide in cacodylate buffer for 2 hours and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate. They were photographed through a Zeiss 109 electron microscope. It should be noted for comparison purposes that the *S. typhimurium* shown in the electron micrograph of Figure 12-B appear similar to those shown previously in intestinal epithelial cells following an experimental infection of the mouse, Takeuchi, 1967, Am. J. Pathol. 50:109-1361.

Shown in Figure 12-B are numerous *Salmonella typhimurium*, denoted by arrows, in extracellular spaces as well as contained within a single cell, possibly a neutrophil, seen in the upper left. Also seen in the field are two unidentified cells that appear to be dying as indicated by the large intracellular space, along with cellular debris.

18. EXAMPLE: ATTENUATION OF *SALMONELLA TYPHIMURIUM* BY MUTATION TO AUXOTROPHY

The studies below demonstrate that the reduced virulence of clone 72 (see, *e.g.*, Section 15.2 above) is due to a Pur^- phenotype. Further described are analyses of avirulent derivatives of clone 72 that were isolated as additional auxotrophic mutants, expressing in different combinations the phenotypes of Ade^-, Ilv^-, Arg^-, Aro^-, and Ura^-.

18.1. MUTATION TO AUXOTROPHY

Clone 72 was examined for auxotrophic mutations and was found to have growth requirements for both adenine and vitamin B1, indicating a mutation(s) in the purine biosynthetic pathway (Pur^-). An experiment was designed to

test whether the ade$^-$ mutation could account for the observed attenuation of clone 72 described above. Populations of both wild type strain 14028 and clone 72 were mutagenized with UV radiation and nitrosoguanidine as described in Section 7.1. From the population of mutagenized strain 14028, three separate Pur$^-$ auxotrophic mutant clones were isolated and designated clones N, Q, and T. From the population of mutagenized clone 72, three separate Pur$^+$ revertant clones were isolated and designated clones R, U, and W.

C57B/6 mice were injected i.p. with 2 x 10^6 c.f.u. *Salmonella typhimurium* of each of the strains obtained. The mice were allowed to eat and drink *ad libitum* and the cages were monitored for dead or moribund mice. Moribund animals (listless, cessation of drinking) were euthanized and counted with the other dead. After 10 or 30 days post-injection with bacteria the surviving animals were euthanized.

The results are shown in Table 20(B).

TABLE 20(B)

SURVIVAL OF C57B6 MICE INJECTED WITH DIFFERENT AUXOTROPHIC MUTANTS OF *SALMONELLA TYPHIMURIUM*

Strain	Phenotype	Time of Death (Days ± S.D.)	Survivors >10 days	Survivors >30 days
14028	wild type	3.0 ± 0.5	n.a.	n.a.
72	superinfective, ade$^-$	5.8 ± 1.4	n.a.	n.a.
R	72, Pur$^+$	3.9 ± 0.4	n.a.	n.a.
U	72, Pur$^+$	3.9 ± 1.3	n.a.	n.a.
W	72, Pur$^+$	4.1 ± 0.9	n.a.	n.a.
T	14028, Pur$^-$	6.8 ± 1.5	n.a.	n.a.
N	14028, Pur$^-$	n.a.	4/8	n.d.
Q	14028, Pur$^-$	n.a.	5/8	n.d.
YS721	72, Ilv$^-$	n.a.	10/11	6/11
YS7211	72, Ilv$^-$, Arg$^-$	n.a.	8/8	7/8
YS7213	72, Ilv$^-$, Aro$^-$	n.a.	8/8	8/8
YS7212	72, Ilv$^-$, Ura$^-$	n.a.	8/8	6/8

Results are the average ± SD for n = 8-12 animals
n.a., not applicable; n.d., not done.

As shown in Table 20(B), Clone 72 was less virulent than the wild type strain 14028. However, 3 of 3 Pur$^+$ revertants of clone 72 (U, W, and T) expressed virulence similar to 14028. Conversely, 3 of 3 Pur$^-$ auxotrophic mutants isolated from strain 14028 (T, N, and Q) were less virulent than either 14028 or clone 72.

Isolation of additional auxotrophs from clone 72 produced even less virulent strains. For example, clone YS721 is an isoleucine-valine requiring (Ilv^-) derivative of clone 72, and clone YS721 was significantly less virulent than clone 72. Similarly, auxotrophic derivatives of clone YS721 such as clones YS7211 (Arg^-), YS7212 (Ura^-), and YS7213 (Aro^-) were all significantly less virulent than YS721 itself.

18.2. EVIDENCE THAT THE SUPERINFECTIVE PHENOTYPE OF CLONE 72 IS GENETICALLY DISTINCT FROM ITS AUXOTROPHIC PURINE REQUIREMENT

The various *Salmonella typhimurium* Pur^- and Pur^+ strains described above in Section 18.1 were assayed for their ability to infect human M2 melanoma cells in culture. The *in vitro* infection assay employed was as described in Section 18.1.

The results are described in Table 20(C).

TABLE 20(C)

INFECTIVITY TOWARD HUMAN M2 MELANOMA CELL *IN VITRO* BY VARIOUS PURINE MUTANTS OF *SALMONELLA TYPHIMURIUM*

Strain	Phenotype	Infecting *Salmonella*/ 10^6 melanoma cells/15'(±S.D.)	x wild type
14028	wild type	$1.0 \pm 0.2 \times 10^5$	1.0x
72	superinfective, ade^-	$9.8 \pm 0.7 \times 10^5$	9.8x
R	72, Pur^+	$5.9 \pm 1.4 \times 10^5$	5.9x
U	72, Pur^+	$1.1 \pm 0.2 \times 10^6$	11x
W	72, Pur^+	$1.1 \pm 0.3 \times 10^6$	11x
N	14028, Pur^-	$1.9 \pm 0.5 \times 10^5$	1.9x
Q	14028, Pur^-	$1.5 \pm 1.0 \times 10^5$	1.5x
T	14028, Pur^-	$1.1 \pm 0.4 \times 10^5$	1.5x

Results are the average ± SD for triplicate infections. The bacteria were cultured in LB broth to $O.D._{600} = .600$ prior to their dilution and use in the infection assays.

As shown in Table 20(C), Clone 72 displayed superinfectivity toward human M2 melanoma cells compared to wild type strain 14028. None of the 14028 Pur⁻ derivatives differed significantly in its infectivity from strain 14028 itself, and all of the clone 72 Pur⁺ derivatives expressed superinfectivity similar to clone 72 itself. The results demonstrate that the purine requirement exhibited by clone 72 which accounts for the reduced virulence of clone 72 in mice, is genetically separate from the superinfective phenotype of clone 72. These results demonstrate that neither mutation to nor reversion from purine auxotrophy effects expression of the superinfective phenotype characteristic of clone 72.

18.3. RETENTION OF THE SUPERINFECTIVE PHENOTYPE BY ATTENUATED DERIVATIVES OF *SALMONELLA TYPHIMURIUM* CLONE 72

In the experiments below, the infectivity of certain auxotrophic derivatives of clone 72 described above in Section 18.1 was assessed *in vitro*. The phenotypes of the clones of *Salmonella* evaluated are shown in Table 20(B) in Section 18.1 above. Infectivity assays described in Section 10.1 were employed.

The results are presented in Table 20(D).

TABLE 20(D)

INFECTIVITY OF *SALMONELLA TYPHIMURIUM* AUXOTROPHS TOWARD HUMAN MELANOMA CELLS IN CULTURE

Strain	Infecting *Salmonella*/ 10^6 melanoma cells/15 min	x wild type
14028 (wild type)	$4.3 \pm \times 10^4$	1.0x
clone 72	$4.4 \pm \times 10^5$	10x
clone YS721	$3.2 \pm \times 10^5$	7.4x
clone YS7211	$2.0 \pm \times 10^5$	4.7x
clone YS7212	$1.7 \pm \times 10^5$	4.0x
clone YS7213	$1.3 \pm \times 10^3$	0.03x

The results represent the average ± SD for 10-19 separate infections. The bacteria were grown in LB broth to $O.D._{600} = 0.5$ before being diluted prior to their use in the infection assays

Salmonella typhimurium clones YS721, YS7211, and YS7212, though each somewhat less infective of M2 melanoma cells than clone 72, were nonetheless superinfective when compared to wild type strain 14028, indicating their partial retention of the superinfective phenotype. In contrast, clone YS7213 (Ade$^-$, Ilv$^-$, Aro$^-$) was found to have greatly reduced infectivity, being about 30-fold less infectious toward M2 melanoma cells than the wild type strain 14028.

18.4. **GROWTH OF PUR$^-$ AND URA$^-$ MUTANTS OF *SALMONELLA TYPHIMURIUM* WITH NUTRITIONAL ADDITIVES OR EXTRACTS OF B16F10 MELANOMA**

Tumor extracts were prepared in the following manner: B16F10 melanoma tumor cells (5 x 10^5) were implanted s.c. into 68 week female C57B6 mice. After 3-4 weeks, the mice were sacrificed and the tumors removed aseptically and rapidly frozen, -20°C. A total of 51 g of frozen pooled tumors was thawed at 4°C and vigorously homogenized in 255 ml (5 vol) H_2O in a capped Virtis tissue homogenizer in the cold for 1 hour. The resulting homogenate was made 10% with trichloracetic acid (TCA), placed on ice for 15 minutes, and centrifuged in a Beckman J21 centrifuge at about 20,000x g for 15 minutes at 4°C. Further procedures were conduced at room temperature. The clear, colorless supernatant fraction (300 ml) was retained and extracted by manual shaking for 1 minute with 1 volume (300 ml) anhydrous ether. Between extractions, the mixtures were allowed to settle and the upper phase (containing ether, extracted TCA, as well as ether-soluble compounds from the tumor extract) was removed by aspiration and discarded through approved environmentally-protective procedures. During 5 such extraction cycles, the pH of the water phase rose from a starting value of about pH 1 to a final value of pH 4-5, similar to that of distilled H_2O, indicating that the TCA had been effectively removed. The water phase was bubbled with a stream of nitrogen for about 15 minutes, at which time the odor of ether had disappeared.

The solution was then filtered through a 0.2 micron filter, divided into aliquots and either used directly in the assays herein, or stored at -20°C for further use.

Wild type strain 14028, and its auxotrophic derivatives clone 72 (Pur^-, vitamin $B1^-$), and YS7212 (Ade^-, vitamin $B1^-$, Ilv^-, Ura^-) were grown overnight on a slant in 5 ml Luria broth (LB) at 35°C. The next day 0.1 ml of each culture was diluted into 10 ml of Medium 56 (0.037 M KH_2PO_4, 0.06 M Na_2HPO_4, 0.02% $MgSO_4$-$7H_2O$, 0.2% $(NH_4)_2SO_4$, 0.001% $Ca(NO_3)_2$ and 0.00005% $FeSO_4$-$7H_2O$) supplemented with 0.2 μg/ml vitamin B1, 33 μg/ml adenine, 50 μg/ml uracil, 83 μg/ml isoleucine, 83 μg/ml valine and 0.3% glucose, and grown on a rotor overnight at 37°C. The next day the cultures were collected by centrifugation and resuspended in plain Medium 56 (1 ml culture plus 9 ml Medium 56). Aliquots (0.25 ml) of these suspensions were then added to Medium 56 containing various supplements in the following manner:

A. Medium 56 plus glucose;

B. Medium 56 plus glucose, vitamin B1, adenine, isoleucine, valine, and uracil; and

C. Medium 56 and tumor extract (10%).

The bacteria were placed in a swirling H_2O bath, 37°C, and growth as a function of OD_{600} was followed with a spectrophotometer. The starting optical densities for all of the cultures ranged from 0.005-0.07.

As demonstrated in Figure 15A-C, wild type strain 14028 was able to proliferate at about the same rates in all three of the media tested, including the most basic of the three, Medium 56 plus glucose. Unlike the wild type strain, neither clone 72 nor clone YS7212 was able to grow in Medium 56 plus glucose, indicative of their nutritional requirements originally observed through replicated plating on agar. In contrast both clone 72 and clone YS7212 were able to grow in Medium 56 supplemented with 10% tumor extract. Liver extracts prepared in the same manner were also able to support the growth of clones 72 and YS7212.

Although the inventors do not wish to be limited to a specific mechanism of action, since the growth state of auxotrophic strains of *Salmonella* is dependent upon the availability of nutrients, such auxotrophs would seem to have advantages as tumor vectors since the environment of the tumor could in theory provide such nutrients, for example in necrotic spaces or within actively dividing cells of the tumor. Thus, mutation of organisms such as *Salmonella* to auxotrophy not only reduces their virulence *in vivo* but also may provide a potential mechanism for their selective population and amplification within solid tumors.

18.5. PROLIFERATION OF PUR⁻ AND URA⁻ MUTANTS OF *SALMONELLA TYPHIMURIUM* IN HUMAN M2 MELANOMA CELLS IN CULTURE

In this Section it is demonstrated that the internal milieu of cultured M2 melanoma cells also can also supply the auxotrophic requirements of these clones, since both clone 72 and clone YS7212 were able to undergo several rounds of division once they invaded M2 melanoma cells cultured under aerobic conditions.

Salmonella typhimurium clones 72 and YS7212 were grown to $O.D._{600}=0.8$, or about 10^9 c.f.u./ml. The two strains were then added at 10^6c.f.u./ml culture media of human M2 melanoma cells as described above in Section 7.2. 15 minutes after infection with *Salmonella*, the eukaryotic cell cultures were rinsed with fresh medium and medium containing gentamicin (10 μg/ml) was added. At hourly intervals over a 6 hour period, cultures were processed as described in Section 7.2 for quantitation of *Salmonella*/10^6 melanoma cells. In addition, control flasks without melanoma cells but with bacteria were processed side-by-side with the experimental flasks containing melanoma cells.

The results are shown in Figure 15-D. Control flasks with *Salmonella* but without melanoma cells showed no viable bacteria over the 6 hour period, demonstrating that the wash procedure coupled with gentamicin treatment

successfully eliminated all viable bacteria not protected by location within animal cells. In contrast, in the presence of M2 melanoma cells, *Salmonella typhimurium* clones 72 and YS7212 each increased significantly in number over the 6 hour period with doubling times of about 2 hours for each strain. Phase and electron microscope analyses demonstrated that M2 melanoma cells were able to compartmentalize infecting *Salmonella* within vacuoles. The results indicate that the net rate of growth of *Salmonella* within the melanoma cells was a steady-state function, reflecting the ability of the melanoma cells to both stimulate growth of the auxotrophs through the supply of nutritional requirements, as well as to suppress the growth of the auxotrophs through anti-bacterial mechanisms.

18.6. **BIODISTRIBUTION OF AUTOTROPHIC ATTENUATED STRAINS OF *SALMONELLA TYPHIMURIUM* IN C57B6 MICE-BEARING B16 MELANOMA TUMORS**

These studies demonstrate the ability of clones YS721, YS7213, YS7211 and YS7212 to target tumors and proliferate within the tumor *in vivo*.

C57B6 6-8 week old female mice were inoculated s.c. (left flank) with 2.5-5.0 x 10^5 B16F10 mouse melanoma cells. When the tumors reached about 0.5 g (14-16 days post-tumor inoculum), the animals were further inoculated i.p. with the indicated strains of *S. typhimurium*. The bacterial inoculum was 4 x 10^5 cfu/mouse for strains 14028 and 72 and 2-4 x 10^6 cfu/mouse for strains YS721, YS7211, YS7212 and YS7213. After 40 and 96 hours post-inoculation of bacteria, the mice were sacrificed, the tumors and livers were removed aseptically, rinsed with sterile NaCl (0.9%), weighed, and homogenized in LB broth at a ratio of 5:1 (vol:tumor wt). Bacteria were quantitated by plating the homogenates onto LB plates, incubating overnight at 37°C, and counting bacterial colonies. The results presented in Table 20(E) represent the average ± SD for n=4-7 animals.

TABLE 20(E)

BIODISTRIBUTION OF WILD TYPE AND ATTENUATED STRAINS OF *SALMONELLA TYPHIMURIUM* IN C57B6 MICE-BEARING B16 MELANOMA TUMORS

	Salmonella/g tissue:		
Strain	Tumor	Liver	Tumor: Liver
A. 40 hrs post-inoculation of bacteria			
14028	$6.5\pm6.8 \times 10^9$	$2.4\pm2.8 \times 10^7$	270:1
72	$1.7\pm1.2 \times 10^9$	$1.9\pm2.3 \times 10^5$	9000:1
YS721	$8.7\pm3.1 \times 10^8$	$4.2\pm3.6 \times 10^6$	210:1
YS7211	$3.3\pm3.0 \times 10^7$	$8.1\pm8.4 \times 10^5$	41:1
YS7212	$3.9\pm7.3 \times 10^7$	$1.1\pm0.8 \times 10^6$	35:1
YS7213	$1.5\pm2.8 \times 10^8$	$4.0\pm3.1 \times 10^5$	375:1
B. 96 hrs post-inoculation with bacteria			
14028	moribund/dead		
72	moribund/dead		
YS721	$3.2\pm1.5 \times 10^9$	$4.7\pm6.9 \times 10^6$	680:1
YS7211	$1.6\pm2.2 \times 10^9$	$6.3\pm9.9 \times 10^6$	253:1
YS7212	$1.1\pm7.4 \times 10^9$	$5.1\pm8.6 \times 10^5$	2200:1
YS7213	$1.3\pm2.5 \times 10^9$	$2.2\pm6.9 \times 10^5$	5900:1

Each of the strains tested was able to target the tumor tissue and replicate to varying degrees within the tumor, as evidenced by the finding that in all cases the tumors contained 10-1000 times more *Salmonella typhimurium* than were first inoculated. Further, in all cases the tumor:liver ratio of bacteria/g tissue was at least 35:1 and in some cases approached 10^4. The tumors analyzed in the studies presented in Table 20(E) ranged in weights from 0.5-2.0 g. Of all the conditions and strains tested, clone 72 exhibited the highest tumor:liver ratio 40 hours post inoculation. Further, *Salmonella typhimurium* strain 14028, as well as clone 72 and its derivatives were also able to target and amplify within larger B16F10 melanoma tumors of, for example, 4-8 g. In addition, as shown in Section 10.3.2

and Table 12A, clone 72 can target and amplify within human solid tumors as small as 40 mg.

However, both clone 72 and the wild type strain 14028 were highly virulent toward C57B6 mice, especially mice bearing tumors. For example, C57B/6 mice bearing B16F10 melanomas injected with strains 1428 and 72 had average survival times of 2.1 ± 0.4 days (n=6) and 4.7 ± 0.5 days (n=9) post-inoculation of bacteria respectively. The biodistribution of these strains was thus not measured at 96 hours. Likewise, clone YS721, though attenuated compared to 14028 and clone 72, was nonetheless virulent in melanoma-bearing mice. For example, B16F10 melanoma-bearing C57B6 mice injected with clone YS721 had an average survival time of 8.1 ± 0.2 days (n=11). *Salmonella* clones YS7211, YS7212 and YS7213, the least virulent of those examined, each displayed densities of greater than 10^9 cfu bacteria/g tumor 96 hrs post-inoculation with tumor:liver ratios of 253:1, 2200:1, and 5900:1 respectively.

18.7. PHENOTYPIC STABILITY FOLLOWING INCUBATION OF *SALMONELLA TYPHIMURIUM* AUXOTROPHS IN TUMOR-BEARING MICE

Genetic reversion of an auxotrophic phenotype could in theory result in an increase in virulence of the previously attenuated bacteria. Therefore, the stabilities of the auxotrophic phenotypes of the strains YS7211, YS7212 and YS7213 were tested following incubation of the bacteria in tumor-bearing mice.

Salmonella typhimurium obtained from the homogenates of livers and tumors of animals 40 hours post-inoculation of either YS7211, YS7212 or YS7213 were picked from LB plates and replicate plated onto minimal media agar plates supplemented with nutritional additives in different combinations. The supplements were isoleucine, valine, adenine/vitamin B1, arginine, uracil, aromatic amino acids, and glucose. For each of the three strains, 50/50 of the bacterial clones recovered from the tumor and liver

homogenates displayed the expected phenotypes of the strain originally inoculated, indicating that in this experiment the strains were genetically stable enough not to revert substantially under the conditions tested.

However, it should be noted that the auxotrophic strains employed were not absolutely stable throughout these studies. In some cases genetic revertants were observed, most notably in the YS7211 strain wherein revertants from Arg^- to Arg^+ were observed. For example, in a tumor-bearing mouse inoculated 96 hours earlier with clone YS7211 bearing a thymidine kinase-containing plasmid, 50 out of 50 bacteria isolated from the liver were found to be Pur^-, Ilv^- and Arg^+, indicating that reversion and selective growth of the reverted organisms had occurred within the mouse. The finding that the auxotrophic phenotypes of clones YS7211, YS7212 and YS7213 were relatively stable in mice was supported by the long term survival of mice inoculated with these strains.

18.8. SUPPRESSION OF TUMOR GROWTH AND INCREASED SURVIVAL OF C57B6 TUMOR-BEARING MICE INOCULATED WITH AUXOTROPHIC MUTANTS OF *SALMONELLA TYPHIMURIUM*

C57B6 female mice, 5-7 weeks old, were inoculated s.c. in the left shoulder region with 5×10^5 B16F10 melanoma cells grown in culture. On the 8th day following inoculation of tumor cells, the mice were further inoculated i.p. with $2-4 \times 10^6$ c.f.u. of *Salmonella typhimurium* strains YS721, YS7211, YS7212 or YS7213. Tumor growth was assessed with periodic caliper measurements of tumor length, width and height, and computed as tumor volume in mm^3. Results of tumor growth, shown in Figure 16A-D represent the averages ± SD for 5 animals/group with 5/5 animals surviving. After the point at which one or more animals died within a group, the average tumor sizes of the surviving animals were no longer shown when the data were plotted as shown in Figure 16A-D.

All tumor measurements were stopped after 33 days post implantation of tumor cells, even though 5/5 tumor-

bearing animals treated with clone YS7211 were still alive at this time. The animals were allowed to eat and drink *ad libitum*. Twenty-three days (Experiment #1) or 10 days (Experiment #2) following inoculation of bacteria, both control and bacteria treated mice were given Baytril™ (enrofloxacin, 0.2 mg/ml drinking water) and maintained on this antibiotic for a total of 2 weeks. In Experiment #1 the times at which the mice became moribund (listless, cessation of drinking) or died, were noted. The results are presented in Table 20(F) as the average survival ± SD for the conditions tested. In Experiment #2 animals were sacrificed when the tumor reached 4 g and listed with the other dead as described in Experiment #1. The two different methods for assessing survival accounted for a somewhat shorter survival time for control animals in Experiment #2 (26 days) as compared to Experiment #1 (28 days).

TABLE 20(F)

SURVIVAL OF B16F10 MELANOMA-BEARING C57B6 MICE INOCULATED WITH *SALMONELLA TYPHIMURIUM*

Strain		Time of death post tumor cell inoculation: (Days ± SD)	Treated/Control (T/C)
Control (no bacteria)	Expt 1	28 ± 2	1.0
	2	26 ± 3	1.0
YS7211	1	36 ± 9	1.3
	2	41 ± 10	1.6
YS7213	1	36 ± 5	1.3
	2	38 ± 6	1.5
YS7212	1	51 ± 7	1.8
	2	55 ± 3	2.1

The results represent the average ± SD for 5 animals.

Figure 16A-D shows the average ± SD tumor volumes (mm^3) versus time post inoculation of 5 x 10^5 B16F10 melanoma cells s.c. into C57B/6 mice. All four clones of *Salmonella*, namely clones YS721, YS7211, YS7212 and YS7213, elicited suppression of tumor growth in the animals. Clone YS721, attenuated through Ade^- and Ilv^- auxotrophy, was nonetheless toxic to tumor-bearing mice and resulted in no prolongation of survival compared to control tumor-bearing animals receiving no bacteria. Whereas the death of control animals was clearly due to very large tumor masses (4-8 g), the death of tumor-bearing animals inoculated with clone YS721 appeared to be a result of bacterial toxicity since the tumor burden in these animals was quite small and not life-threatening in itself. The tumors ranged from non-palpable to less than 0.5 g at the time of death.

In contrast, treatment of tumor-bearing mice with clone YS7211, YS7212 and YS7213, each less virulent than clone YS721, resulted in significant enhancement of survival in addition to suppression of tumor growth. The degree of suppression of tumor growth by the individual *Salmonella* clones, as seen in Figure 16A-D, correlated with their abilities to elicit enhanced survival, as seen in Table 20(F). The average time for tumors to reach 1 g (1000 mm^3) was about 18 days for control animals, 31 days for animals treated either with YS7213 and YS7211 and 45 days (extrapolated) for YS7212. This corresponded to average survival times for 26 days for control tumor-bearing animals, compared to 38, 41, and 55 days for animals treated with clones YS7213, YS7211 and YS7212.

Thus, among the attenuated strain of *Salmonella* tested, the order of efficacy for suppression of tumor growth and prolongation of survival was YS7212>YS7211>YS7213. Earlier treatment with an antibiotic, enrofloxacin, *i.e.*, 10 days as compared to 23 days post-inoculation of bacteria, increased the survival time for tumor-bearing animals inoculated with *Salmonella*, but not that of control animals.

18.9. ANTI-TUMOR ACTIVITY OF AUXOTROPHIC *SALMONELLA TYPHIMURIUM* EXPRESSING CYTOSINE DEAMINASE

Experimental metastasis model of B16F10 was set up by injecting 1 x 10^5 cells into C57B/6 mice via the lateral tail vein on Day 0. Aliquots of 0.2 ml bacterial suspension of YS7212 carrying the cytosine deaminase expression construct (see Figure 4E for the CD construct) (approximately 1 x 10^7 CFU/ml) were injected intraperitoneally into mice on Day 5. 5-Flourocytosine (5-FC), at 0.4 ml aliquots dissolved in PBS at 10 mg/ml (final dose: 200 mg/kg), was injected into mice intraperitoneally on Day 7. Death of animals was recorded daily. Results are presented in Figure 17.

Figure 17 clearly demonstrates that combination of CD and 5-fluorocytosine prolong the survival *Salmonella* expressing animals bearing B16F10 lung metastases.

19. EXAMPLE: ATTENUATION OF *SALMONELLA TYPHIMURIUM* THROUGH MUTATION IN LIPOPOLYSACCHARIDE BIOSYNTHESIS

Several mutant strains of *Salmonella typhimurium* and *E. coli* have been isolated with genetic and enzymatic lesions in the LPS pathway (Raetz, 1993, J. Bacteriol. 175:5745-5753). One such mutant, the *firA*$^-$ mutation is within the gene that encodes the enzyme UDP-3-O(R-30 hydroxymyristoyl)-glycocyamine N-acyltransferase, that regulates the third step in endotoxin biosynthesis (Kelley et al., 1993, J. Biol. Chem. 268:19866-19874). *Salmonella typhimurium* and *E. coli* strains bearing this type of mutation produce a lipid A that differs from wild type lipid A in that it contains a seventh fatty acid, a hexadecanoic acid (Roy and Coleman, 1994, J. Bacteriol. 176:1639-1646) and has decreased lipid A 4' kinase activity.

A *firA*$^-$ mutant was investigated for its ability to induce TNFα production by human monocytes as well as its ability to target solid tumors in mice.

19.1. ABILITY OF *SALMONELLA TYPHIMURIUM firA*⁻ TO INDUCE TNF-α PRODUCTION BY HUMAN BLOOD MONOCYTES

Salmonella typhimurium strain SH5014 and its *firA*⁻ derivative SH7622 are described in Hirvas et al., 1991, EMBO J. 10:1017-1023. The genotypes of these strains are as follows:

strain SH5014 *ilv*-1178 *thr*-914 *his*-6116 *met*A22 *met*E551 *trp*B2 *xyl*-404 *H1-b H2-e,n,x fla*A66 *rps*L120 *rfa*J4041;

strain SH7622 *ilv*-1178 *thr*-914 *his*-6116 *met*A22 *met*E551 *trp*B2 *xyl*-404 *H1-b H2-e,n,x fla*A66 *rps*L120 *rfa*J4041, *ssc-1*(*firA*^{ts}).

A derivative of *Salmonella typhimurium firA*⁻ strain SH7622 was picked, designated SH7622-64, and used as the *firA*⁻ strain for the experiments in this section as well as in Section 19.2 below. SH7622-64 was selected for its supersensitivity to the antibiotic novobiocin and temperature-sensitive growth, characteristics of the *firA*⁻ SH7622 strain.

LPS was extracted from *Salmonella typhimurium* strain 14028 and its derivatives clone 72, clone YS7212, and clone YS7213; as well as strain SH5014 and its *firA*⁻ derivative, clone SH7622-64, as follows: The bacteria were grown in 500 ml LB broth to $O.D._{600}$=0.9 or about 2 x 10^9 cfu/ml. They were collected by centrifugation, and the pellets, containing about 10^9 cfu/ml. They were collected by centrifugation, and the pellets, containing 10^{12} bacteria, were drained and stored frozen at -20°C. To extract LPS, the pellets were resuspended in 18.3 ml H_2O, and 15 ml redistilled phenol was added (H_2O:phenol, 55:45, vol/vol). The mixtures were placed in a shaking water bath at 69-70°C, for 1 hour producing a monophasic mixture, and then cooled on ice. On cooling the mixture separated into a phenol phase containing mainly proteins, and a water phase containing lipopolysaccharide and nucleic acid (Galanos, C., Luderitz, O., and Westphal, O., 1969). The water phase was lyophilized to dryness and the white fluffy lyophilized material was used as the source of LPS. The LPS was weighed and dissolved in

H_2O at 1 mg/ml, as stock for dilution in the incubations with human macrophages described below.

Human macrophages were prepared as follows and all procedures were at room temperature: Blood (60 ml) was collected from a healthy human volunteer into a heparinized syringe. The blood was layered in 7 ml aliquots over 4 ml of Isolymph™ (density - 1.077 g/ml; 9.0 g sodium diatrizoate and 5.7 g Ficoll 400™/100 ml H_2O; Pharmacia Fine Chemicals, A.B. Uppsala, Sweden) in 15 ml Corning Plastic Centrifuge tubes, centrifuged at 2000x g for 45 minutes. The red blood cells pelleted through the Isolymph™, neutrophils and other cells sedimented in a discrete band above this interface, and above the lymphocyte/macrophage band was serum, visible by its yellow color. The serum from each tube was removed by pipette, pooled in a total volume of about 30 ml and saved for supplementation into the culture media as described below. The lymphocyte/macrophage bands were pooled in a total volume of about 15 ml diluted with 40 ml RPMI 1640 culture medium, and centrifuged at 1000x g for 5 minutes. The cloudy supernatant was discarded and about 0.2 ml of pelleted white cells was obtained. The cells were resuspended with 50 ml RPMI 1640 culture medium supplemented with 15% human serum (described above), penicillin (100 units/ml) and streptomycin (100 μg/ml). The recovery of viable lymphocytes and macrophages from 60 ml whole blood was determined by hemocytometer counting to be about 7×10^7 cells. Together the cells, lymphocytes and monocytes were distributed into 24 well Corning Tissue Culture Plates at 0.5 ml/well, and incubated in a gassed humidified incubator at 37°C for 15 hours.

The next day the cultures were rinsed twice with serum-free RPMI 1640 containing antibiotics. Between each rinse, the cultures were incubated about 1 hour in the 37°C incubator to facilitate removal of lymphocytes and other non-adherent cells. Adherent cells were found to be mostly, if not all derived from blood monocytes, *i.e.*, macrophages that had differentiated from their blood monocyte state by virtue

of attachment to the culture dish. For example, in a histochemical assay to determine the percentage of macrophages in the adherent population of cells 48 hours post-plating into culture, the population was found to be essentially 100% positive for expression of the enzyme non-specific esterase, a marker commonly used to distinguish monocytes and macrophages from lymphocytes and other cell types. Results indicated that most if not all of the cells employed in the LPS challenge described below were of monocyte origin.

After the second rinse described above, serum-free, antibiotic containing RPMI 1640 supplemented with LPS at the concentrations indicated was added to the cells, and the cultures were placed in a gassed, humidified incubator at 37°C overnight. After 20 hours, the well plate cultures were centrifuged in a Beckman GS-15 centrifuge at 8000x g for 10 minutes, and the supernatants were removed and assayed for TNF-α content using the QUANTIKINE™ Human TNF-α Immunoassay Kit #DTA50 (R&D Systems, Minneapolis, MN). TNF-α production as pg/ml by human macrophages is plotted as a function of pg/ml bacterial LPS added to the culture medium and shown in Figure 18.

Strain 14028 and its derivatives clone 72, clone YS7212, and clone YS7213, as well as strain SH5014, all induced TNF-α production by human macrophages at concentrations of LPS in a dose-dependent fashion. Concentrations of LPS from each of these strains as low as 100 pg/ml (0.1 ng/ml) were stimulatory to TNF-α production, and increasingly stimulatory at concentrations of 10^3 pg/ml and 10^4 pg/ml, inducing TNF-α production by macrophages to levels of 600-800 pg/ml. The levels of TNF-α induced by the LPS were similar to the circulating levels of TNF-α found in patients with septic shock syndrome as well as in human volunteers injected with *E. coli* LPS (Morrison et al., 1994, ASM News 60:479-484). In contrast, LPS from *firA*⁻ strain SH7622-64 was far less stimulatory to TNF-α production by the macrophages, and was detected only at concentrations of

10^4 pg/ml. Thus, on a dose response comparison, LPS from strain SH7622-64 was only about 1% as effective in stimulating macrophage TNF-α production when compared to LPS from the *firA*$^+$ parental strain SH5014. Furthermore, the results demonstrate that strains YS7212 and YS7213 each produced LPS similar to wild type strain 14028 LPS, as assessed by stimulation of human macrophages.

19.2. TUMOR TARGETING BY *SALMONELLA TYPHIMURIUM* BEARING THE *firA*$^-$ MUTATION

M27 mouse lung tumor cells or B16F10 mouse melanoma cells (5×10^5) were implanted s.c. in C57B6 mice. When the tumors were palpable, SH7622-64 grown in LB broth at 37°C to a density of about 10^9 cfu/ml (OD_{600}=0.8). Aliquots of 5-10 x 10^6 cfu were removed and inoculated into tumor bearing mice. At 48 hrs (M27 lung) and 96 hrs (B16F10 melanoma) post-inoculation of bacteria the animals were sacrificed, the tumors and livers removed, weighed and homogenized in LB broth at a ratio of 5 ml/g tissue. Homogenates were quantitated for bacteria by serial dilutions on LB agar plates. Results are presented in Table 20(G).

TABLE 20(G)

TUMOR LOCATION BY *SALMONELLA TYPHIMURIUM* BEARING THE *firA*$^-$ MUTATION FOR LIPOPOLYSACCHARIDE BIOSYNTHESIS

Primary Tumor	*Salmonella/g* tissue: Tumor	Liver	Tumor: Liver
M27 lung	2.9×10^6	-0-	n.a.
B16	3.2×10^5	1×10^2	3200:1

The results are derived from single animals.

As shown in Table 20(G), strain SH7622-64 was able to locate within both the B16F10 melanoma and the M27 lung tumors when inoculated i.p. into mice. These results, in combination with those in Section 19.1 which show that LPS from this particular *firA*⁻ strain was greatly suppressed in its ability to induce TNF-α in human macrophages, demonstrate that *Salmonella* attenuated through a mutation in endotoxin biosynthesis can be useful as tumor vectors *in vivo*.

20. EXAMPLE: TUMOR-SPECIFIC ACCUMULATION OF CLONES YS721 AND YS7211 IN MURINE LEWIS LUNG CARCINOMA

This example demonstrates that auxotrophic mutant *Salmonella* clones YS721 and YS7211 locate to lung carcinoma.

The experimental model of the Lewis lung carcinoma was set up by injecting $5x10^5$ cells into C57B/6 mice subcutaneously on Day 0. Aliquots of 0.2 ml bacterial suspension (approximately 1 x 10^7 CFU/ml) were injected intraperitoneally into mice on Day 14. On Day 16, the tumors and livers were harvested and homogenized and bacterial counts determined by plating serial dilutions. Results of the relative distribution are shown in Table 20(H).

TABLE 20(H)

TUMOR SPECIFIC ACCUMULATION OF CLONES YS721 and YS7211 IN MICE

Strain	No. pathogens /g Liver	No. pathogens /g Tumor	Ratio: tumor/liver
YS721	9.8×10^6	4.7×10^{10}	4.8×10^3
	4.3×10^5	3.2×10^{10}	7.3×10^4
	1.1×10^6	1.4×10^9	1.3×10^3
	1.6×10^6	1.0×10^{12}	6.2×10^5
	3.0×10^4	2.3×10^9	7.7×10^4
YS7211	1.4×10^4	2.6×10^{10}	1.9×10^6
	1.9×10^5	2.7×10^8	1.4×10^3
	2.3×10^5	6.0×10^{11}	2.6×10^6
	1.0×10^6	5.0×10^{11}	5.0×10^5

Extremely high levels of bacteria were localized to these tumors, as well as others indicating that the auxotrophic mutations retain tumor specific accumulation of bacteria for a spectrum of tumor models.

21. **EXAMPLE: TREATMENT OF B16F10 MELANOMA METASTATIC TUMORS**

Metastases constitute one of the major problems for treatment of solid tumors. While larger tumors can be detected and removed surgically, smaller metastases constitute the untreated reservoir which is frequently the cause of death. Therefore, an effective cancer therapeutic should be effective against metastatic tumors.

An experimental metastasis model of B16F10 was set up by injecting $1x10^5$ CELLS into C57B/6 mice via the lateral tail vein on Day 0. Aliquots of 0.2 ml YS7211/p5-3 and YS7212/p5-3 (YS7211 and YS7212 each carrying the *HSV* thymidine kinase expression plasmid) bacterial suspensions (approximately 1×10^7 CFU/ml) were injected intraperitoneally into mice on Day 5. Ganciclovir, at 0.1 ML aliquots

dissolved in PBS at 22 mg/ml (final dose: 100 mg/kg), was injected into mice intraperitoneally on Day 7. Tumor progression was monitored by periodic sacrifice and examination of the lungs. At day 28, all the animals were sacrificed and the normal and tumor-bearing lungs weighted.

Figure 19 clearly demonstrates that animals inoculated with YS7212 carrying the *HSV* thymidine kinase gene (YS7212/p5-3) and further treated with GCV show reductions in the number and extent of B16F10 lung metastases.

22. EXAMPLE: DIAGNOSIS OF TISSUE BIOPSIES FOR MELANOMA USING *SALMONELLA TYPHIMURIUM*

Diagnosis of melanoma according to the methods of the present invention can be performed using, for example, *Salmonella typhimurium* as follows: A portion of a biopsied specimen suspected of melanoma is minced with scissors in tris-buffered saline (TBS) and then incubated in Ca^{++}/Mg^{++} - free saline containing trypsin, collagenase, and EDTA (Sigma Chemicals) for 60 minutes at 37°C to dissociate the tissue into individual cells. The cells are then rinsed free of the dissociation enzymes by centrifugation. The cells are resuspended in 1 ml DMEM/10% FBS and added to a 24 well Corning tissue culture chamber containing cover slips in the wells. The cells are then incubated in a gassed (5% CO_2/95% air) humidified incubator for 3 hours at 37°C to allow for attachment to the cover slip.

After attachment of the biopsied cells is achieved, an attenuated, super-infective, melanoma-specific strain of *Salmonella typhimurium* (10^6-10^7 c.f.u./ml) is added. The bacteria and biopsied cells are incubated together at 37°C for 15 minutes to allow for infection of melanoma cells by the *S. typhimurium*, and the cells are then rinsed with TBS to remove non-infecting bacteria. The cells are then permeablized with 0.01% saponin in 3% bovine serum albumin for 5 min, stained for DNA for 10 minutes with 2.5 mg/ml 4'-6 Diamidino-2-phenyhndole (DAPI) and saponin (.01%) in TBS, washed with TBS, mounted in Mowiol (Calbiochem) containing 1,

4-Diazabicyclo (2,2,2) octane (DABCO, Kodak) and observed by phase and fluorescence microscopy. The presence of DAPI-stain in the cytoplasm of the biopsied cells would indicate that they were melanoma cells, *i.e.*, cells that were infected by the melanoma-specific *S. typhimurium* are melanoma cells rather than melanocytes.

23. EXAMPLE: MELANOMA TUMOR TARGETING BY *LISTERIA MONOCYTOGENES*

This example demonstrates that *Listeria monocytogenes* targets to and proliferates in tumor cells when administered to melanoma bearing animals.

C57B/6 mice were inoculated s.c. in the left flank with 5 x 10^5 B16F10 melanoma cells. When the tumors reached about 1-2 g (16 days post implantation of tumor cells) the animals were inoculated i.p. with 7 x 10^5 cfu of *Listeria monocytogenes* wild type strain 43251. Prior to inoculation into mice, the *Listeria* culture was grown overnight in LB media to an OD_{600} of 0.25. At the times indicated animals were sacrificed and the tumors and livers were removed, homogenized and quantitated for bacterial numbers by plating serial dilutions onto L.B. plates.

Tumors were analyzed at 24, 48, and 96 hours post-inoculation of *Listeria monocytogenes*. Results are shown in Table 20(I).

TABLE 20(I)

AMPLIFICATION OF *LISTERIA MONOCYTOGENES* IN C57B/6 MICE-BEARING B16F10 MELANOMA TUMORS

Time Post-inoc.	*L. monocytogenes*/g tissue: Tumor	Liver	Tumor: Liver
24 hrs	$1.5 \pm 1.4 \times 10^3$	$8.0 \pm 6.1 \times 10^4$	1:5
48 hrs	$6.3 \pm 8.5 \times 10^2$	$1.3 \pm 1.0 \times 10^5$	1:210
96 hrs	$5.2 \pm 6.8 \times 10^5$	$5.7 \pm 9.0 \times 10^5$	1:1

The results represent the average ± SD of triplicate determinations.

As shown in Table 20(I) it was found that the levels of bacteria within the tumors rose about 100 fold during this time period, indicating that wild type *Listeria monocytogenes* can target tumors and proliferate within them. *Listeria monocytogenes* strain 43251 was virulent in the C57B/6 mice, causing death about 5 days pot-inoculation i.p. of 7×10^5 cfu.

24. EXAMPLE: *LEISHMANIA AMAZONENSIS* SHOWS TUMOR CELL SPECIFICITY

24.1. *LEISHMANIA AMAZONENSIS* SPECIFICALLY ATTACHES TO HUMAN MELANOMA CELLS *IN VITRO*

Leishmania amazonensis trypomastigotes are regarded as being highly biospecific, in that they are unable to infect virtually any cell types other than macrophages. Since human melanomas are known to express some macrophage-like traits it was determined whether *Leishmania amazonensis* would be able to enter into human melanoma cells in culture. *Leishmania amazonensis* promastigotes were grown in Schneider's Drosophila media (GIBCO BRL) containing 15% heat-inactivated fetal calf serum at 24°C until the parasites were in late log phase (usually 3 to 4 days). Animal cells used

in the *L. amazonensis* infection assays were a mouse melanoma cell line which forms non-metastatic tumors when injected into C57B6 mice (B16/F1), two human metastatic melanoma cell lines (M2 and M2-A7, and as a negative control human foreskin fibroblasts, HFF. These different cell types were grown on glass coverslips in 24 well plates or on plastic Lab-Tec® (Nunc) slides in MEM culture medium with 10% fetal calf serum, for HFF cells; Ham's F10 medium with 10% horse serum for B16/F1 cells; and DMEM with 10% fetal calf serum buffered with 10 mM HEPES, for M2 and M2-A7 cells.

The *Leishmania* parasites were pre-incubated for about one hour with 5% normal human serum and the cultured cells were infected with 0.5 to 5.0×10^6 parasites/ml for about two hours at 32°C. After incubation the cells were washed twice with phosphate buffered saline (PBS) and fixed with 3% paraformaldehyde for about 30 minutes at about 4°C. An anti-*Leishmanial* antibody was incubated with the fixed cells at a normal working dilution (1: 100,000) in PBS with 3% bovine serum albumen (BSA) for about one hour. After washing, a fluorescent-conjugated anti-mouse antibody (Boehringer Mannheim) was incubated with the cells at a normal working dilution (1:500) for about one hour and then washed from the cells. The cells were then permeabilized with 0.02% Saponin (Sigma, a detergent used to remove lipids, thereby allowing penetration by antibodies) in Tris buffered saline (TBS) for 10 min and stained for DNA with 5.0 mg/ml DAPI stain (Sigma) in TBS with 0.02% Saponin. The cells were washed with TBS and mounted on glass slides using Mowiol (CalBiochem) with DABCO (Kodak, a compound that sustains fluorescent emissions). The presence of internalized parasites was determined by failure to react with an anti-*Leishmania* monoclonal antibody in the absence of host cell membrane permeabilization.

Observations made immediately after addition of the live parasites using an inverted phase microscope showed that among these cell lines, motile parasites were immediately adherent only when they encountered the metastatic melanoma

cells, suggesting that the metastatic cells may possess an appropriate receptor for *Leishmania*. These results are shown in Figure 13.

To determine whether or not the *Leishmania* parasites were internalized, M2 human melanoma cells were grown, infected with *Leishmania* and fixed as described above with 3% paraformaldehyde at 4°C for about 30 minutes without permeabilization, washed and immunostained with a monoclonal antibody directed toward a *Leishmania* surface protein, followed by a rhodamine-conjugated anti-mouse antibody. After washing in Tris-buffered saline (TBS), the cells were permeablized with 0.01% saponin in 3% BSA for 5 minutes, and stained for DNA 10 minutes with 2.5 mg/ml 4'-6 Diamidino-2-phenylindole (DAPI) and 0.01% saponin in TBS, washed with TBS, mounted in Mowiol (Calbiochem) containing 1, 4-Diazabicyclo (2,2,2) octane (DABCO, Kodak) and observed by phase and fluorescence microscopy. This procedure detects all parasites and distinguishes between those which are internalized (inaccessible to antibody staining in non-permeabilized cells), and those which are attached but not internalized.

Parasites were internalized by M2 cells (data not shown). Internalization was estimated to occur in 3% of the melanoma cells. These findings demonstrate that a) live *Leishmania* parasites were able to enter the melanoma cells, and b) possibly only a sub-population of the melanoma cells were involved in the process.

24.2. LYSOSOMAL FUSION FOLLOWS INTERNALIZATION OF *LEISHMANIA* BY MELANOMA

In the normal course of invasion of macrophages by *Leishmania*, lysosomes fuse with the phagosome. To determine whether or not this also occurs when *Leishmania* invade melanoma cells, parasites were co-localized with a lysosomal glycoprotein (lgp) marker. The cells were grown, infected and fixed as described above except the cells were immunostained with a monoclonal antibody directed against a

human lysosomal glycoprotein, LAMP-1, followed by a rhodamine-conjugated antimouse antibody. After washing in TBS, the cells were stained for DNA 10 minutes with 2.5 mg/ml DAPI, washed with TBS, mounted in Mowiol containing DABCO and observed by phase and fluorescence microscopy. Parasites co-localizing with LAMP-1 are shown in Figures 14A-C. Co-localization corroborates the internalization of the parasite and demonstrates that the process of lysosomal fusion occurs when *Leishmania* is internalized into the melanoma cells. In summary, *Leishmania amazonensis* in its wild type state shows invasion ability for human melanoma cells that has been heretofore unreported.

25. **EXAMPLE: DIAGNOSIS OF MELANOMA IN HUMAN TISSUE BIOPSIES USING *LEISHMANIA AMAZONENSIS***

Diagnosis of melanoma according to the methods of the present invention can be performed using, for example, *Leishmaina Amazonensis* as follows: A portion of a biopsied specimen suspected of melanoma is minced with scissors in tris-buffered saline (TBS) and then incubated in Ca^{++}/Mg^{++}-free saline containing trypsin, collagenase, and EDTA at 37°C for 60 minutes to dissociate the tissue into individual cells. The cells are then rinsed free of the dissociation enzymes by centrifugation. The cells are resuspended in 1 ml DMEM/10% FBS and added to a 24 well Corning tissue culture chamber containing cover slips in the wells. The cells are then incubated in a gassed, 5% CO_2, humidified incubator at 37°C for about three hours to allow for attachment to the cover slip.

After attachment of the biopsied cells is achieved, a melanoma-specific strain of *Leishmania amazonensis* promastigotes which has been isolated according to the methods of the present invention is added. The parasites and biopsied cells are incubated together at 37°C for about two hours to allow for infection of melanoma cells by the *Leishmania amazonensis* and the cells are then rinsed with TBS to remove non-infecting parasites. The cells are then

permeablized with 0.01% saponin in 3% bovine serum albumin for five minutes, stained for DNA with 2.5 mg/ml 4'-6 Diamiclino-2-phenylindole (DAPI) and saponin (.01%) in TBS for 10 minutes, washed with TBS, mounted in Mowiol (Calbiochem) containing 1,4-Diazabicyclo (2,2,2) octane (DABCO, Kodak) and observed by phase and fluorescence microscopy. The presence of DAPI-stain in the cytoplasm of the biopsied cells would indicate that they were melanoma cells.

26. EXAMPLE: DIAGNOSIS OF HUMAN TISSUE BIOPSIES FOR MELANOMA USING *MYCOBACTERIUM AVIUM*

Mycobacterium avium were found associated with the melanoma cells but not with the normal melanocytes. This discriminatory ability for melanoma cells demonstrates the ability of *Mycobacterium avium* as a vector in the diagnosis and treatment of melanoma. Diagnosis of melanoma using *Mycobacterium avium* is as follows: A portion of a biopsied specimen suspected of melanoma is minced with scissors in tris-buffered saline (TBS) and then incubated in Ca^{++}/Mg^{++}-free saline containing trypsin, collagenase, and EDTA at 37°C for 60 minutes to dissociate the tissue into individual cells. The cells are then rinsed free of the dissociation enzymes by centrifugation. The cells are resuspended in 1 ml DMEM/10% FBS and plated onto 12mm glass cover slips in 24 well plates with $1x10^5$ cells per well. The cells are then incubated in a gassed 5% CO_2, humidified incubator 37°C for 3 hours to allow for attachment of the cells to the cover slip.

After attachment of the biopsied cells is achieved, a melanoma-specific strain of *Mycobacterium avium* which has been isolated by the methods of the present invention is added. The bacteria and biopsied cells are incubated together at 37°C for 15 minutes for infection of melanoma cells by the *Mycobacterium avium*. The cells are then rinsed with TBS to remove non-infecting bacteria. The cells are then permeablized with 0.01% saponin in 3% bovine serum albumin for five minutes, stained for DNA with 2.5 mg/ml 4'-6

Diamidino-2-phenylindole (DAPI) and saponin (.01%) in TBS for 10 minutes, washed with TBS, mounted in Mowiol (Calbiochem) containing 1, 4-Diazabicyclo (2,2,2) octane (DABCO, Kodak) and observed by phase and fluorescence microscopy. The presence of DAPI-stain in the cytoplasm of the biopsied cells would indicate that they were melanoma cells.

27. **DEPOSIT OF MICROORGANISMS**

The following microorganisms were deposited with the American Type Culture Collection (ATCC), Rockville, MD on June 1, 1995 and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.
Clone #70	55686
Clone #71	55685
Clone #72	55680
Clone #72^{5-3-2}	97179
Population #72^{pop-1}	55684
Population #72^{pop-2}	55683
Population #14028^{pop-1}	55681
Population #14028^{pop-2}	55682

The following microorganisms were deposited with the American Type Culture Collection (ATCC), Rockville, MD on May 29, 1996, and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.
Clone YS721	_____
Clone YS7211	_____
Clone YS7212	_____
Clone YS7213	

The following plasmids were deposited with the American Type Culture Collection (ATCC), Rockville, MD on May 29, 1996, and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.

pTK-Sec3 _____
pCD-Sec1 _____
pSP-SAD4-5 _____

The invention claimed and described herein is not to be limited in scope by the specific embodiments herein disclosed since these embodiments are intended as illustrations of several aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

A number of references are cited herein, the entire disclosures of which are incorporated herein, in their entirety, by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Pawelek, John M.
Bermudes, David
Low, Kenneth Brooks

(ii) TITLE OF INVENTION: VECTORS FOR THE DIAGNOSIS AND TREATMENT OF SOLID TUMORS INCLUDING MELANOMA

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
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(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE: On Even Date Herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Baldwin, Geraldine F.
(B) REGISTRATION NUMBER: 31,232
(C) REFERENCE/DOCKET NUMBER: 8002-036

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 790-9090
(B) TELEFAX: (212) 869-9741/8864
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATCATGCAT GGCTTCGTAC CCCGGCC 27

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTAGATGCAT CAGTGGCTAT GGCAGGGC 28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTAGACTAGT TTGTCAATAA TGACAACACC C 31

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCGGATCC TTGCCCGGCG CGGCGGCCTG 30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAGAAGCTT ATAAGGGTTG ATCTTTGTTG TC 32

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTACGATATC CAGAACGATG TGCATAGCCT G 31

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Tyr Thr Ser Gly Tyr Ala His Arg Ser
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Gly Tyr Arg Ile Pro
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCATGCAT GTGGAGGCTA ACAGT 25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTAGATGCAT CAGACAGCCG CTGCGAAGGC 30

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 139-140, lines 1-35 of the description '

A. IDENTIFICATION OF DEPOSIT '

Further deposits are identified on an additional sheet '

Name of depositary institution '

American Type Culture Collection

Address of depositary institution (including postal code and country) '

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit ' June 1, 1995 Accession Number ' 55686

B. ADDITIONAL INDICATIONS ' (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ' (If the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS ' (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later ' (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the International application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau '

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

Accession No.	Date of Deposit
55685	June 1, 1995
55680	June 1, 1995
97179	June 1, 1995
55684	June 1, 1995
55683	June 1, 1995
55681	June 1, 1995
55682	June 1, 1995
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996

WHAT IS CLAIMED IS:

1. A biologically pure culture of microorganisms selected from the following strains:

Microorganism	ATCC Accession No.
Salmonella typhimurium strain #70	55686
Salmonella typhimurium strain #71	55685
Salmonella typhimurium strain #72	55680
Salmonella typhimurium strain #72^{5-3-2}	97179
Salmonella typhimurium strain #14028^{pop-1}	55684
Salmonella typhimurium strain #14028^{pop-2}	55683
Salmonella typhimurium strain #72^{pop-1}	55681
Salmonella typhimurium strain #72^{pop-2}	55682
Salmonella typhimurium strain YS721	_____
Salmonella typhimurium strain YS7211	_____
Salmonella typhimurium strain YS7212	_____ and
Salmonella typhimurium strain YS7213	_____.

2. A plasmid encoding the Herpes Simplex Virus thymidine kinase gene designated pTK-Sec3 and having ATCC Accession No. _____.

3. A plasmid encoding the *E. coli* cytosine deaminase gene designated pCD-Sec1 and having ATCC Accession No. _____.

4. A plasmid encoding the human p450 oxidoreductase gene designated pSP-SAD4-5 and having ATCC Accession No. _____.

5. A method of treatment for a solid tumor cancer, comprising:

administering an effective amount of an isolated population of tumor cell-specific microorganisms to a patient having a solid tumor cancer.

6. The method according to claim 5 wherein the isolated population of tumor cell-specific microorganisms is

an isolated population of super-infective, tumor cell-specific microorganisms.

7. The method according to claim 6 wherein the super-infective, tumor cell-specific population is attenuated.

8. The method according to claim 7 wherein the attenuated population expresses an altered lipid A molecule.

9. The method according to claim 7 wherein the attenuated population induces TNF-α expression to a lesser degree compared to a non-attenuated population.

10. The method according to claim 5 wherein the isolated population of tumor cell-specific microorganisms is selected from the group consisting of *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma hominis*, *Rickettsiae quintana*, *Streptococcus* spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp., *Trypanasoma cruzi*, *Encephahtozoon cuniculi*, *Nosema helminthorum* and *Unikaryon legeri*.

11. The method according to claim 5 or 6 wherein the isolated population of super-infective, tumor cell specific microorganisms is selected from the group consisting of *Salmonella typhimurium* strain #14028^{pop-1}, *Salmonella*

typhimurium strain #14028^{pop-2}, *Salmonella typhimurium* strain #72^{pop-1} and *Salmonella typhimurium* strain #72^{pop-2}.

12. The method according to claim 6 wherein the isolated population of super-infective, tumor cell specific microorganisms is produced by:

(a) exposing a cell culture of a solid tumor cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells; and

(b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected cell culture.

13. The method according to claim 5 wherein the isolated population of tumor cell-specific microorganisms is produced by:

(a) exposing a microorganism to tumor cell conditioned medium for a time sufficient to allow the microorganism to chemotact towards the tumor cell conditioned medium; and

(b) isolating a population of microorganisms which chemotacts towards the tumor cell conditioned medium.

14. The method according to claim 6 wherein the isolated population of super-infective, tumor cell specific microorganisms is produced by:

(a) exposing a mammal having a solid tumor cell cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells; and

(b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected tumor cells.

15. The method according to claim 12, 13 or 14 further comprising:

subjecting the microorganism to mutagenesis before step (a).

16. The method according to claim 12, 13 or 14 further comprising:

(c) repeating steps (a) and (b) a desired number of times.

17. The method according to claim 5, 12, 13 or 14 wherein the solid tumor cell cancer is melanoma cancer.

18. The method according to claim 5, 12, 13 or 14 wherein the solid tumor cancer is colon carcinoma cancer.

19. The method according to claim 5, 12, 13 or 14 wherein the soild tumor cancer is selected from the group consisting of lung cancer, liver cancer, kidney cancer, prostate cancer and breast cancer.

20. A method of treatment for a solid tumor cancer, comprising:

administering an effective amount of a single colony clone of an isolated population of tumor cell-specific microorganisms to a patient having a solid tumor cancer.

21. The method according to claim 20 wherein the single colony clone of an isolated population of tumor cell-specific microorganisms is a single colony clone of an isolated population of super-infective, tumor cell-specific microorganisms.

22. The method according to claim 20 wherein the super-infective, tumor cell-specific single colony clone is attenuated.

23. The method according to claim 22 wherein the attenuated clone expresses an altered lipid A molecule.

24. The method according to claim 20 wherein the attenuated clone induces TNF-α expression to a lesser degree compared to a non-attenuated clone.

25. The method according to claim 20 wherein the single colony clone of an isolated population of tumor cell-specific microorganisms is selected from the group consisting of *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma hominis*, *Rickettsiae quintana*, *Streptococcus* spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp., *Trypanasoma cruzi*, *Encephahtozoon cuniculi*, *Nosema helminthorum* and *Unikaryon legeri*.

26. The method according to claim 20 or 21 wherein the single colony clone of an isolated population of super-infective, tumor cell specific microorganisms is selected from the group consisting of *Salmonella typhimurium* strain #70, *Salmonella typhimurium* strain #71, *Salmonella typhimurium* strain #72, *Salmonella typhimurium* strain #72^{5-3-2}, *Salmonella typhimurium* strain YS721, *Salmonella typhimurium* strain YS7211, *Salmonella typhimurium* strain YS7212 and *Salmonella typhimurium* strain YS7213.

27. The method according to claim 21 wherein the single colony clone of an isolated population of super-infective, tumor cell specific microorganisms is produced by:

(a) exposing a cell culture of a solid tumor cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells;

(b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected cell culture; and

(c) culturing the population isolated in step (b) so that single colony clones are obtained.

28. The method according to claim 20 wherein the single colony clone of an isolated population of tumor cell specific microorganisms is produced by:

(a) exposing a microorganism to tumor cell conditioned medium for a time sufficient to allow the microorganism to chemotact towards the tumor cell conditioned medium;

(b) isolating a population of microorganisms which chemotacts towards the tumor cell conditioned medium; and

(c) culturing the population isolated in step (b) so that single colony clones are obtained.

29. The method according to claim 21 wherein the single colony clone of an isolated population of super-infective, tumor cell specific microorganisms is produced by:

(a) exposing a mammal having a solid tumor cell cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells;

(b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected tumor cells; and

(c) culturing the population isolated in step (b) so that single colony clones are obtained.

30. The method according to claim 27, 28 or 29 further comprising:

subjecting the microorganism to mutagenesis before step (a).

31. The method according to claim 27, 28 or 29 further comprising:

(d) repeating steps (a) and (b) a desired number of times before step (c).

32. The method according to claim 20, 27, 28 or 29 wherein the solid tumor cell cancer is melanoma cancer.

33. The method according to claim 20, 27, 28 or 29 wherein the solid tumor cancer is colon carcinoma cancer.

34. The method according to claim 20, 27, 28 or 29 wherein the soild tumor cancer is selected from the group consisting of lung cancer, liver cancer, kidney cancer, prostate cancer and breast cancer.

35. The method according to claim 20 or 21 wherein the single colony clone is genetically engineered.

36. The method according to claim 35 wherein the single colony clone expresses a suicide gene.

37. The method according to claim 36 wherein the suicide gene is encoded by a plasmid selected from the group consisting of pTK-Sec3, pCD-Sec1 and pSP-SAD4-5.

38. The method according to claim 36 wherein the single colony clone expresses a suicide gene selected from the group consisting of p450 oxidoreductase, *HSV* thymidine kinase, *E. coli* cytosine deaminase, carboxypeptidase G2, β-glucuronidase, penicillin-V-amidase, penicillin-G-amidase, β-lactamase, β-glucosidase, nitroreductase and carboxypeptidase A.

39. The method according to claim 37 or 38 wherein the suicide gene is selected from the group consisting of *HSV* thymidine kinase and *E. coli* cytosine deaminase.

40. The method according to claim 36 wherein expression of the suicide gene is controlled by a constitutive promoter.

41. The method according to claim 36 wherein expression of the suicide gene is controlled by an inducible promoter.

42. The method according to claim 36 wherein expression of the suicide gene is controlled by a bacterial promoter that is activated in specific target cells.

43. The method according to claim 42 wherein expression of the suicide gene is controlled by a bacterial promoter that is activated in specific tumor cells.

44. A method of diagnosis for a solid tumor cancer, comprising:

(a) exposing a biopsied sample of cells suspected of being cancer cells to a tumor-specific microorganism for a time sufficient so that the microorganism can infect the cells suspected of being cancerous;

(b) exposing a sample of non-cancerous counterpart control cells to the tumor-specific microorganism for the same time; and

(c) comparing the infectivity of the microorganism for the cells suspected of being cancerous and the non-cancerous counterpart control cells.

45. The method according to claim 44 wherein the microorganism is selected from the group consisting of *Borrelia burgdorferi, Brucella melitensis, Escherichia coli,*

enteroinvasive *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma hominis*, *Rickettsiae quintana*, *Streptococcus* spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp., *Trypanasoma cruzi*, *Encephahtozoon cuniculi*, *Nosema helminthorum* and *Unikaryon legeri*.

46. The method according to claim 44 wherein the microorganism is selected from the group consisting of *Salmonella typhimurium* strain #14028^{pop-1}, *Salmonella typhimurium* strain #14028^{pop-2}, *Salmonella typhimurium* strain #72^{pop-1}, *Salmonella typhimurium* strain #72^{pop-2}, *Salmonella typhimurium* strain #70, *Salmonella typhimurium* strain #71, *Salmonella typhimurium* strain #72, *Salmonella typhimurium* strain #72^{5-3-2}, *Salmonella typhimurium* strain YS721, *Salmonella typhimurium* strain YS7211, *Salmonella typhimurium* strain YS7212 and *Salmonella typhimurium* strain YS7213.

47. The method according to claim 44 wherein the microorganism is produced by:

(a) exposing a cell culture of a solid tumor cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells; and

(b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected cell culture.

48. The method according to claim 44 wherein the microorganism is produced by:

(a) exposing a microorganism to tumor cell conditioned medium for a time sufficient to allow the microorganism to chemotact towards the tumor cell conditioned medium; and

(b) isolating a population of microorganisms which chemotacts towards the tumor cell conditioned medium.

49. The method according to claim 44 wherein the microorganism is produced by:

(a) exposing a mammal having a solid tumor cell cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells; and

(b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected tumor cells.

50. The method according to claim 47, 48 or 49 further comprising:

(c) culturing the population isolated in step (b) so that single colony clones are obtained.

51. The method according to claim 47, 48 or 49 further comprising:

subjecting the microorganism to mutagenesis before step (a).

52. The method according to claim 47, 48 or 49 further comprising:

repeating steps (a) and (b) a desired number of times.

53. The method according to claim 44, 47, 48 or 49 wherein the solid tumor cell cancer is melanoma cancer.

54. The method according to claim 44, 47, 48 or 49 wherein the solid tumor cell cancer is colon carcinoma cancer.

55. The method according to claim 44, 47, 48 or 49 wherein the soild tumor cancer is selected from the group consisting of lung cancer, liver cancer, kidney cancer, prostate cancer and breast cancer.

56. The method according to claim 44 wherein the microorganism is genetically engineered.

57. A diagnostic kit comprising an effective amount of a tumor-specific microorganism.

58. The diagnostic kit according to claim 57 wherein the tumor-specific microorganism is a super-infective, tumor-specific microorganism.

59. The diagnostic kit according to claim 57 further comprising non-cancerous counterpart control cells.

60. The diagnostic kit according to claim 57 wherein the microorganism is selected from the group consisting of *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma hominis*, *Rickettsiae quintana*, *Streptococcus* spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp.,

Trypanasoma cruzi, *Encephahtozoon cuniculi*, *Nosema helminthorum* and *Unikaryon legeri*.

61. The diagnostic kit according to claim 58 wherein the microorganism is selected from the group consisting of *Salmonella typhimurium* strain #14028^{pop-1}, *Salmonella typhimurium* strain #14028^{pop-2}, *Salmonella typhimurium* strain #72^{pop-1}, *Salmonella typhimurium* strain #72^{pop-2}, *Salmonella typhimurium* strain #70, *Salmonella typhimurium* strain #71, *Salmonella typhimurium* strain #72, *Salmonella typhimurium* strain #72^{5-3-2} *Salmonella typhimurium* strain YS721, *Salmonella typhimurium* strain YS7211, *Salmonella typhimurium* strain YS7212 and *Salmonella typhimurium* strain YS7213.

62. The diagnostic kit according to claim 58 wherein the microorganism is genetically engineered.

63. The method according to claims 5, 12, 13 or 14 wherein the solid tumor cancer is a metastatic cancer.

64. The method according to claims 20, 27, 28 or 29 wherein the solid tumor cancer is a metastatic cancer.

65. The method according to claims 44, 47, 48 or 49 wherein the solid tumor cancer is a metastatic cancer.

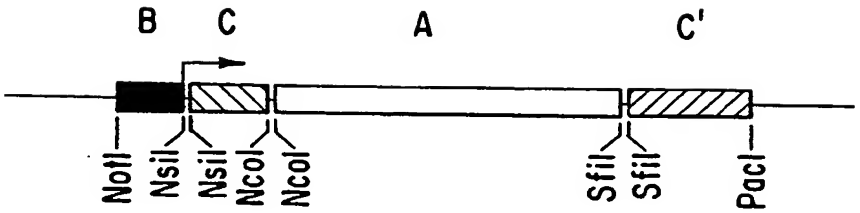

FIG. 1

FIG.2B

FIG.2A

FIG.3A

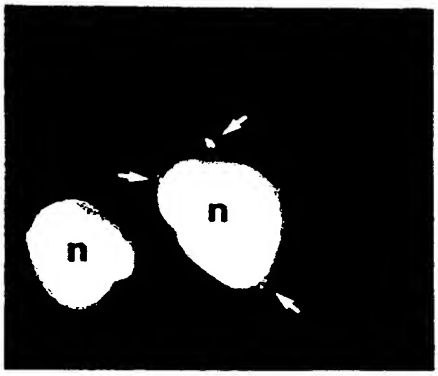

FIG.3B

FIG.3C

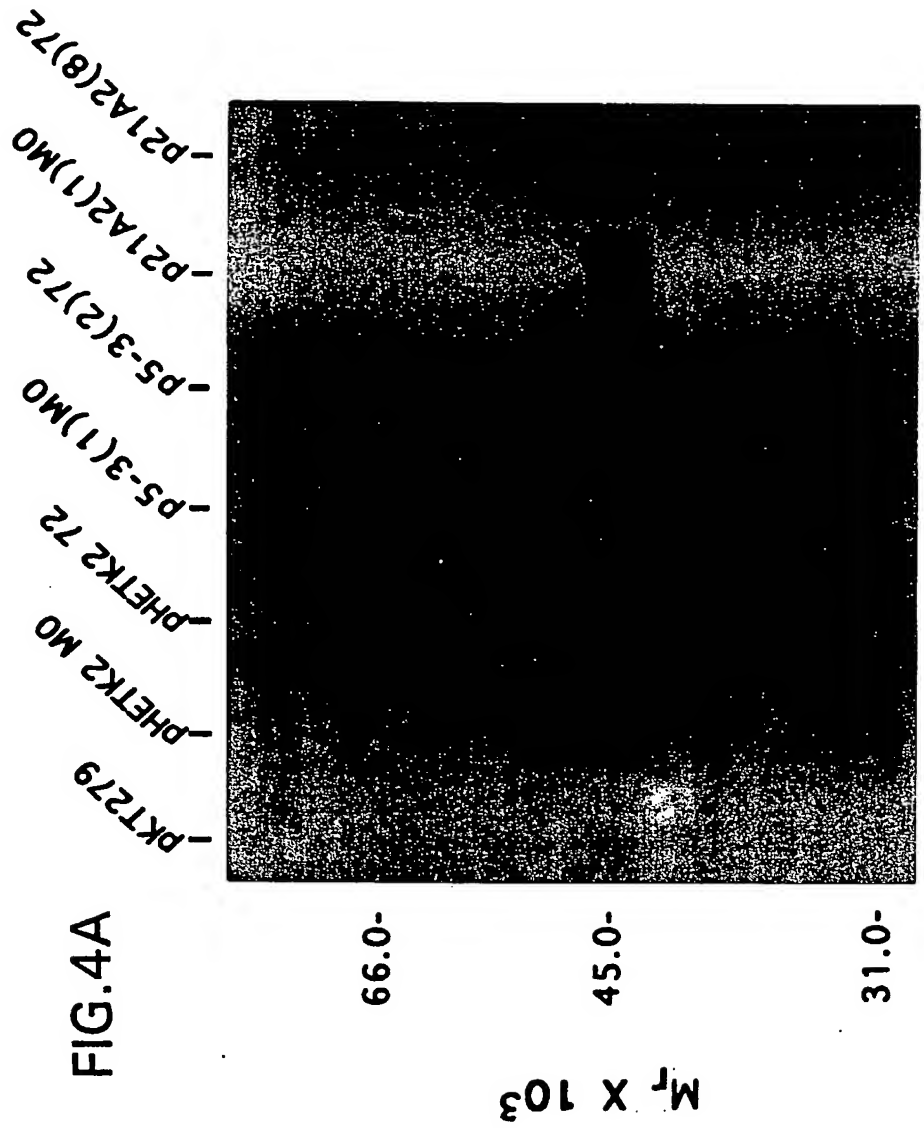
pKT279
pHETK2 MO
pHETK2 72
pS-3(1)MO
pS-3(2)72
p21A2(1)MO
p21A2(8)72
66.0-
45.0-
31.0-
M_r X 10^3

FIG. 4A

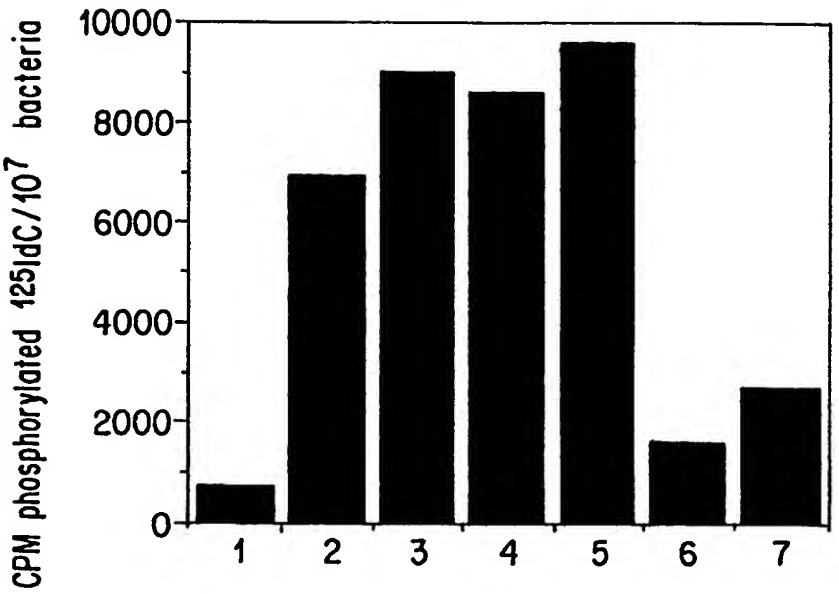
CPM phosphorylated 125IdC/10^7 bacteria
10000
8000
6000
4000
2000
0
1
2
3
4
5
6
7

FIG. 4B

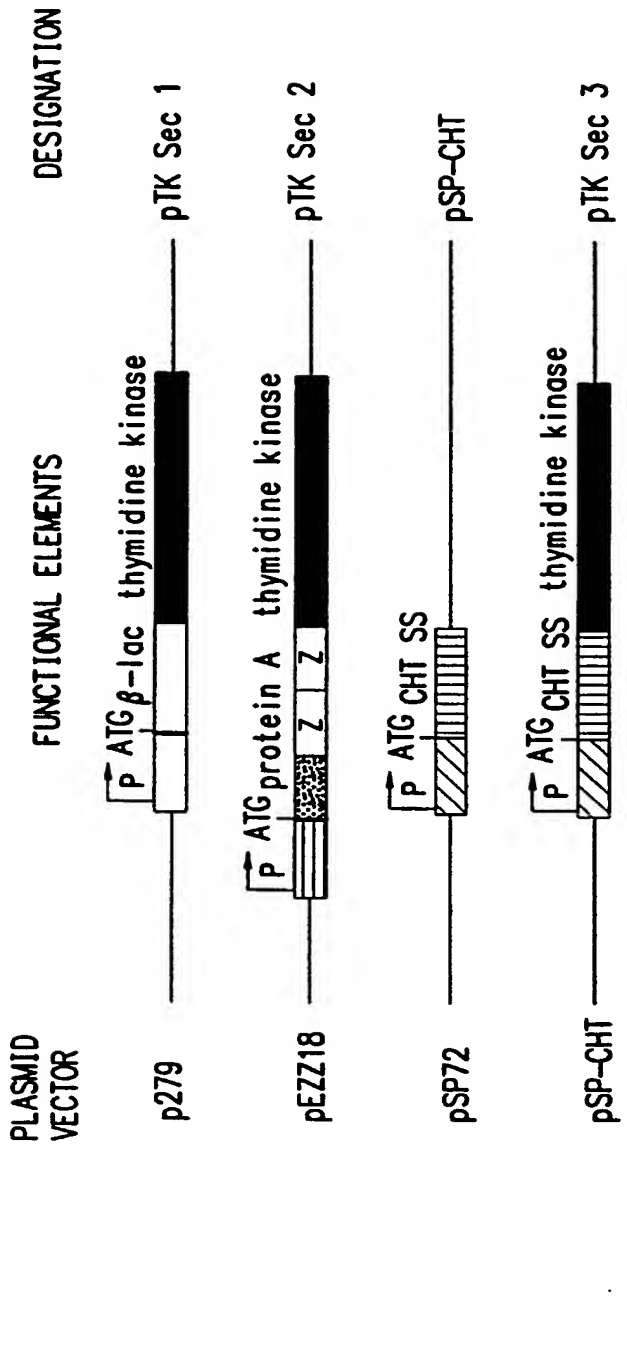
PLASMID VECTOR
FUNCTIONAL ELEMENTS
DESIGNATION
p279
P ATG β-lac thymidine kinase
pTK Sec 1
pEZZ18
P ATG protein A Z Z thymidine kinase
pTK Sec 2
pSP72
P ATG CHT SS
pSP-CHT
pSP-CHT
P ATG CHT SS thymidine kinase
pTK Sec 3
= β-lactamase promoter
= lac I promoter
= chitinase promoter
= protein A signal sequence
= chitinase signal sequence
P = promoter transcriptional initiation
Z = protein A IgG binding domain
= thymidine kinase

FIG.4C

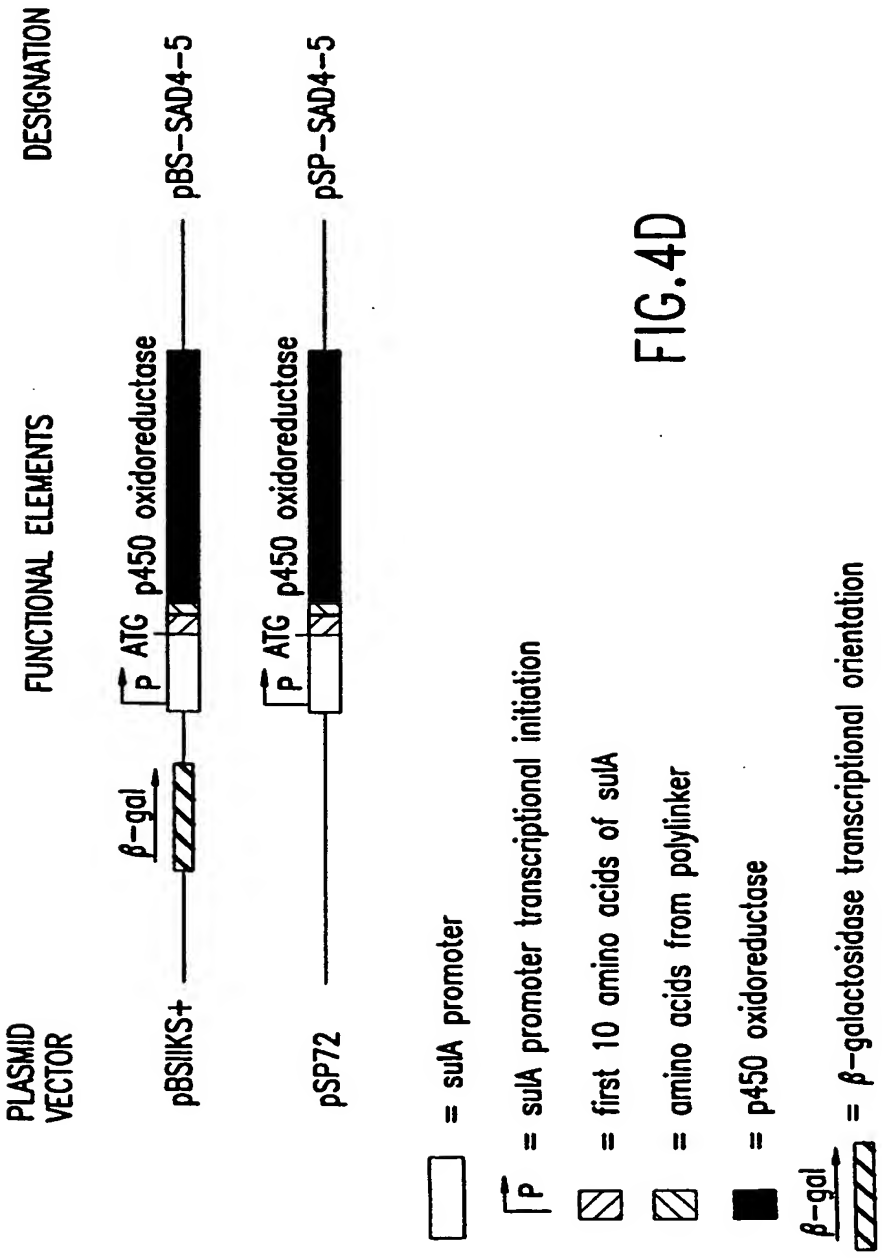
PLASMID VECTOR
FUNCTIONAL ELEMENTS
DESIGNATION
pBSIIKS+
β-gal
P ATG p450 oxidoreductase
pBS-SAD4-5
pSP72
P ATG p450 oxidoreductase
pSP-SAD4-5
= sulA promoter
P = sulA promoter transcriptional initiation
= first 10 amino acids of sulA
= amino acids from polylinker
= p450 oxidoreductase
β-gal = β-galactosidase transcriptional orientation

FIG.4D

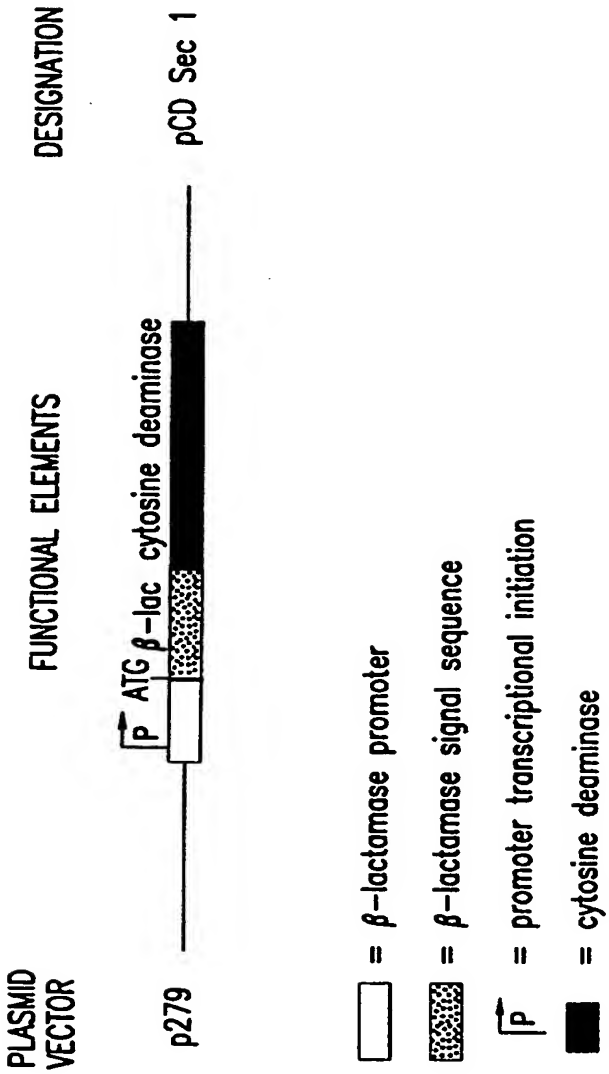
PLASMID VECTOR
FUNCTIONAL ELEMENTS
DESIGNATION
p279
P ATG β-lac cytosine deaminase
pCD Sec 1
= β-lactamase promoter
= β-lactamase signal sequence
P = promoter transcriptional initiation
= cytosine deaminase

FIG. 4E

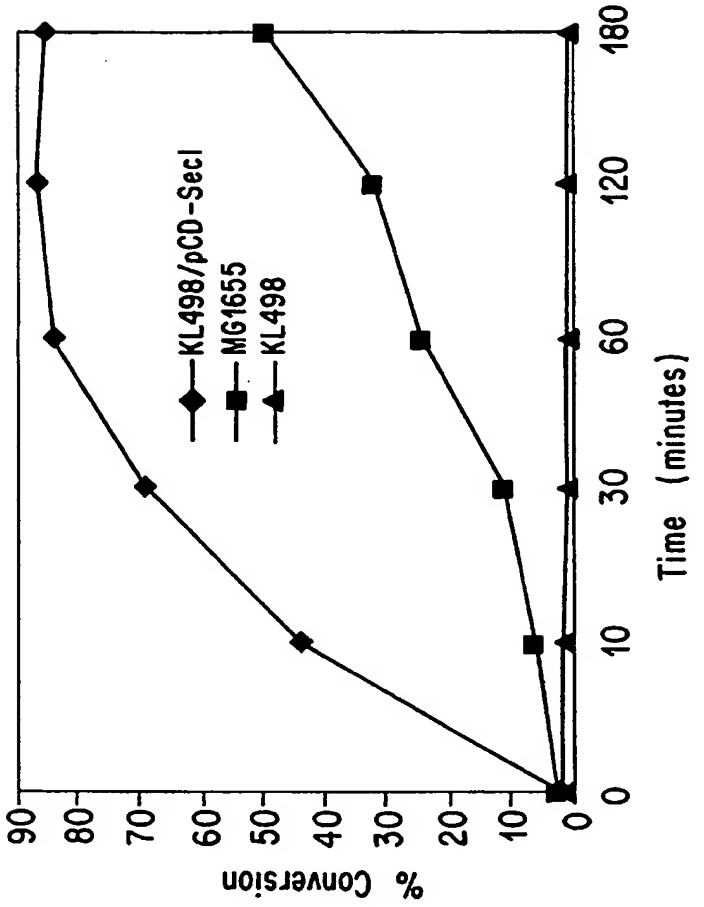
KL498/pCD-SecI
MG1655
KL498
% Conversion
Time (minutes)
0
10
20
30
40
50
60
70
80
90
0
10
30
60
120
180

FIG. 4F

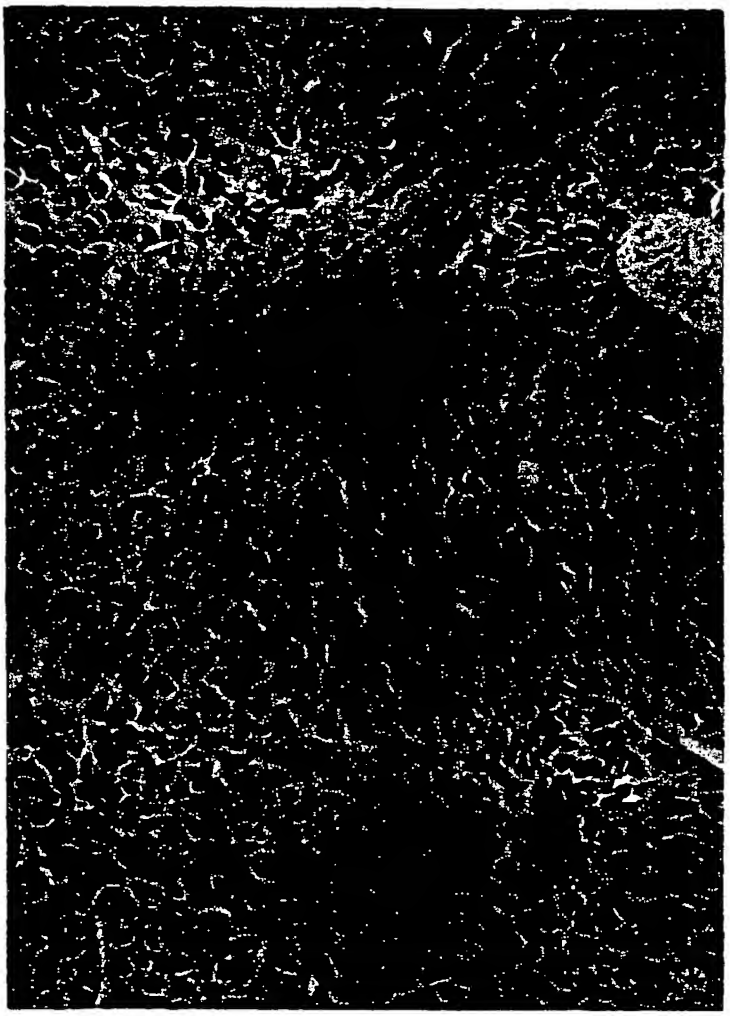

FIG.5A

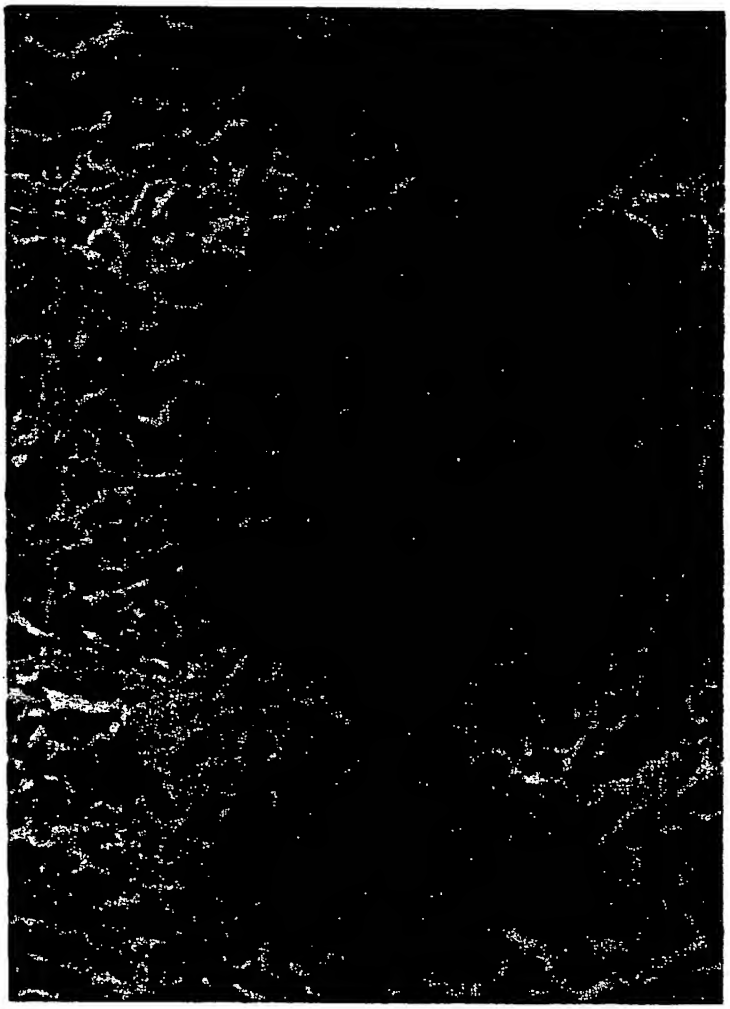

FIG.5B

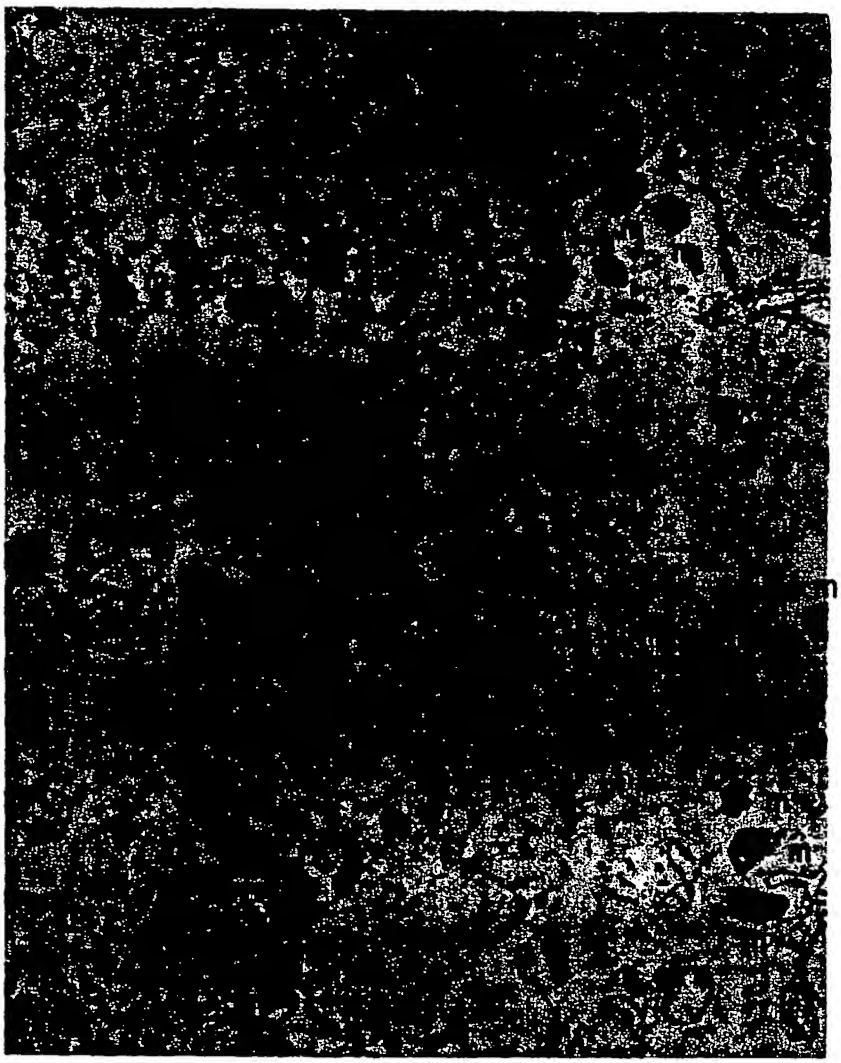

FIG.6

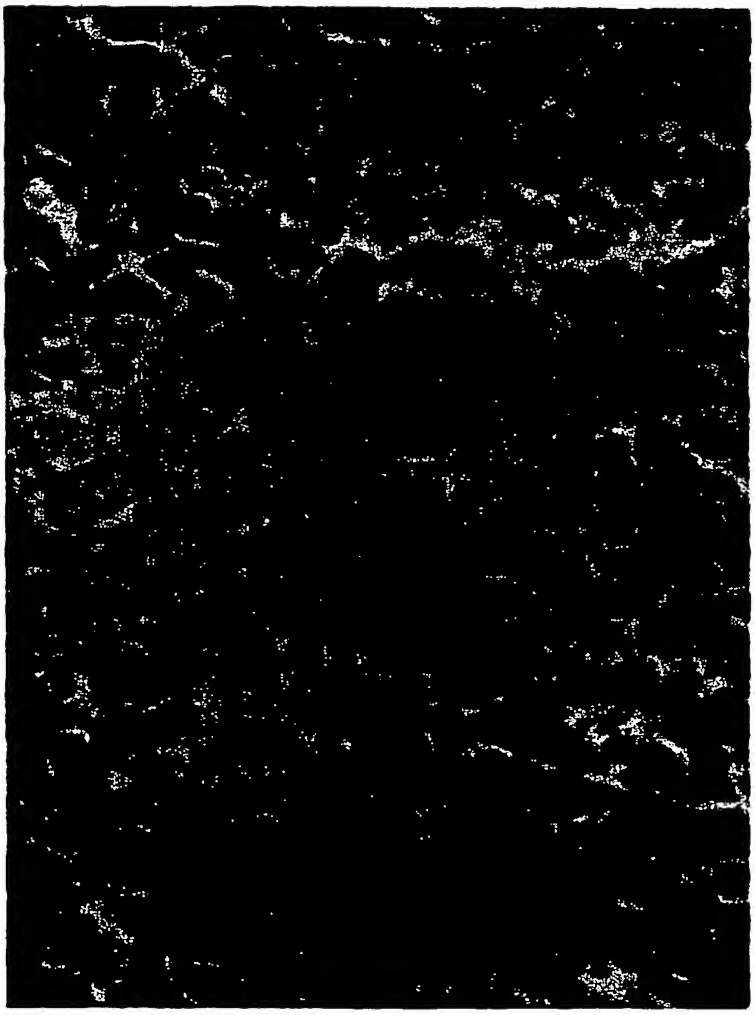

FIG.7

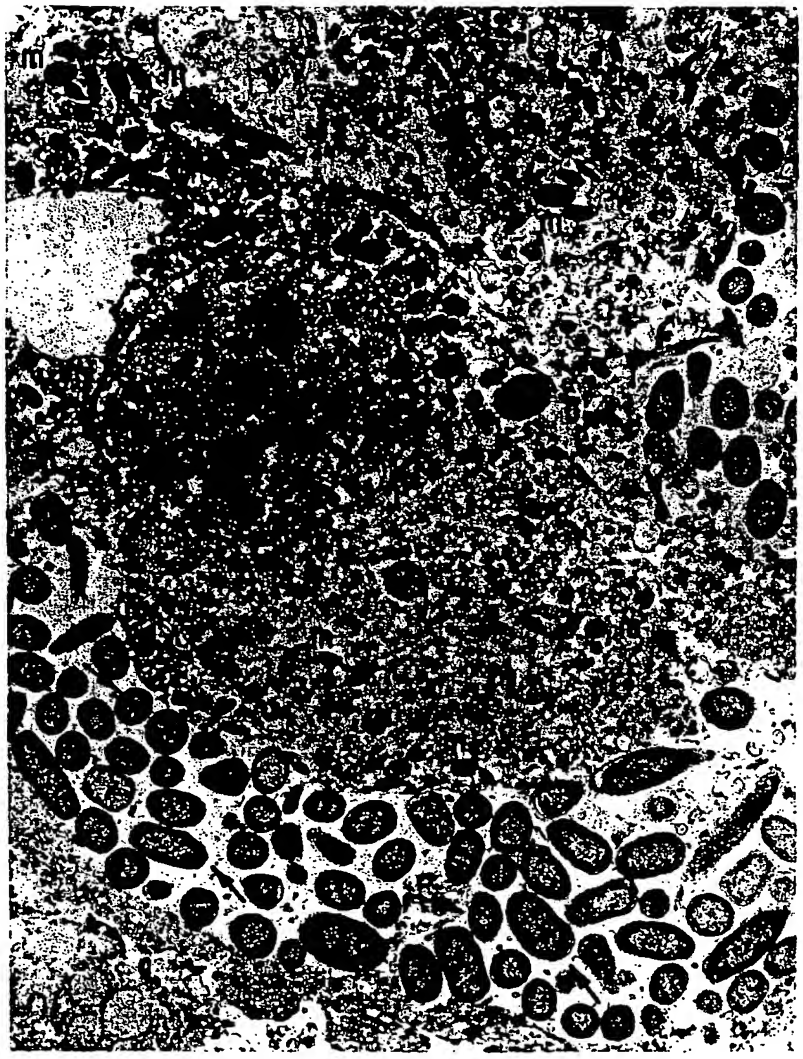

FIG.8

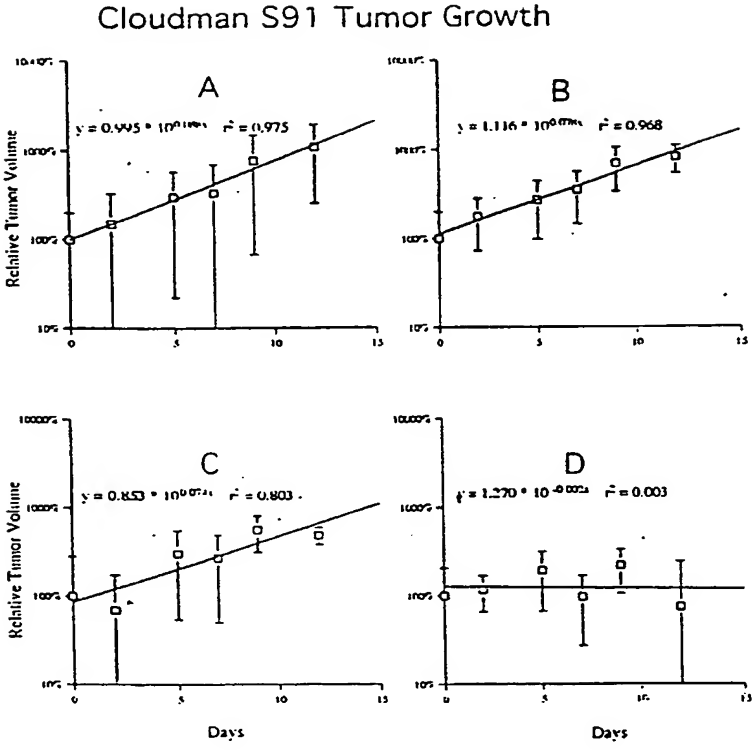
Cloudman S91 Tumor Growth
A
B
C
D
Relative Tumor Volume
Relative Tumor Volume
Days
Days

FIG. 9

CONTROL

FIG.10A

VECTOR (10 DAYS)

FIG.10B

CONTROL + GCV

FIG.11A

TK VECTOR + GCV

FIG.11B

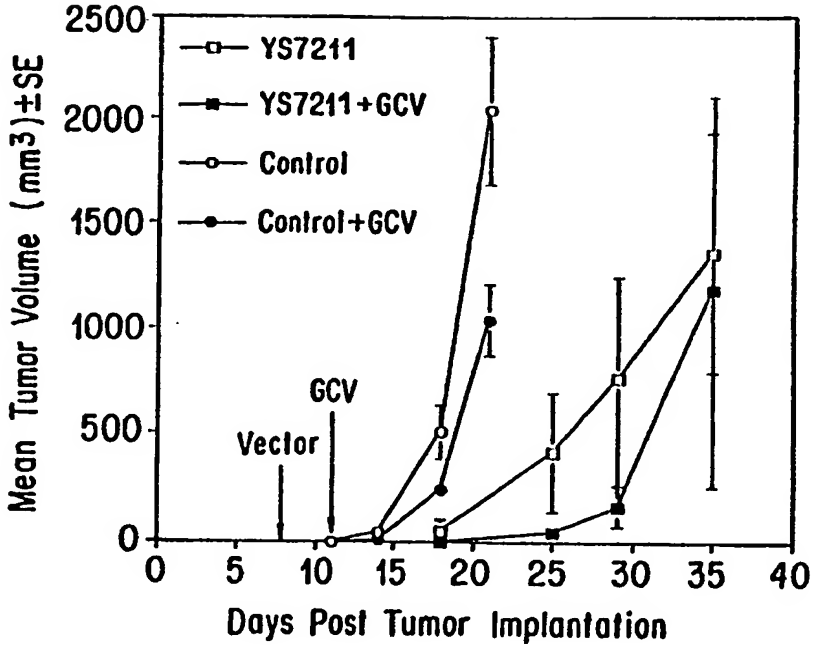

FIG. 11C

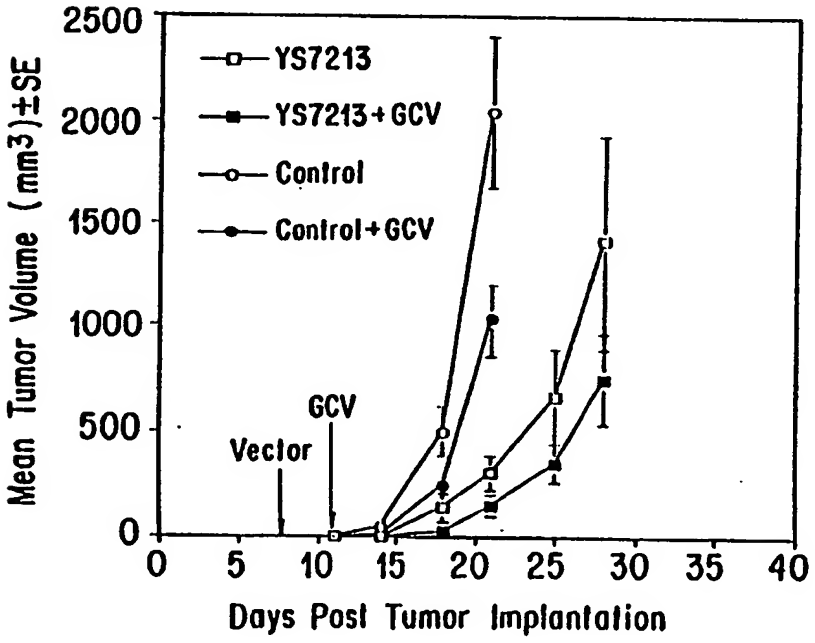

FIG. 11D

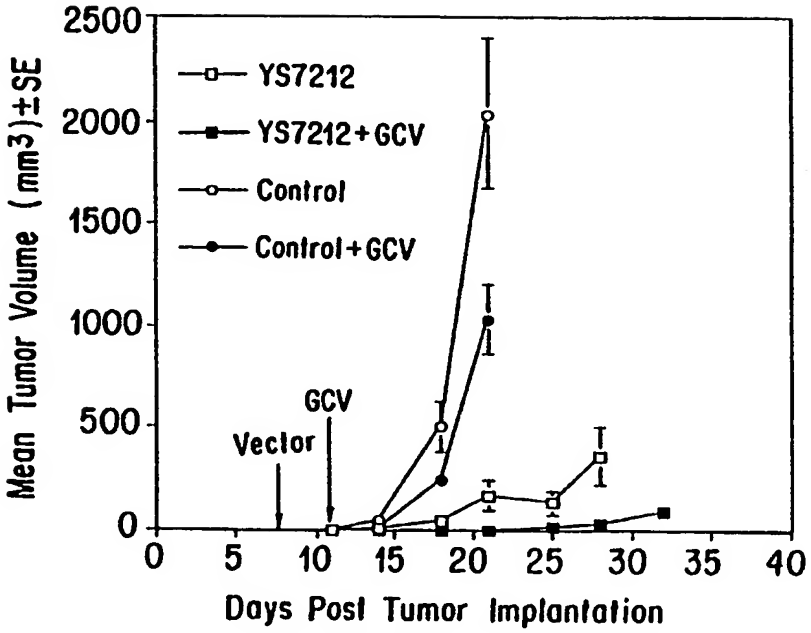
2500
2000
1500
1000
500
0
Mean Tumor Volume (mm3)±SE
YS7212
YS7212+GCV
Control
Control+GCV
GCV
Vector
0
5
10
15
20
25
30
35
40
Days Post Tumor Implantation

FIG.11E

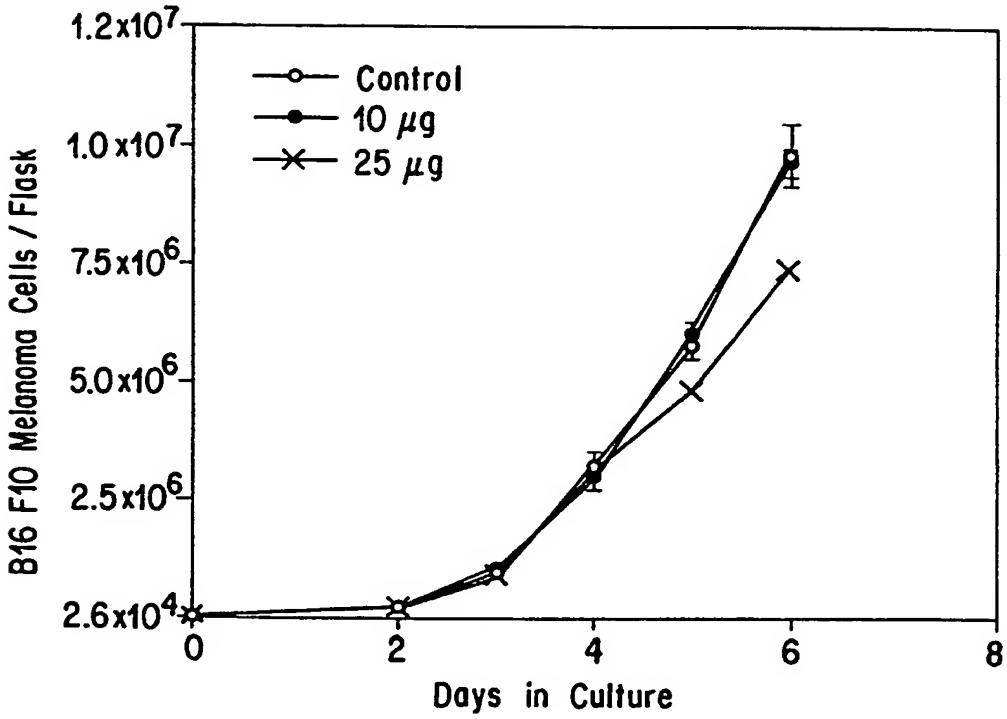
1.2x10^7
1.0x10^7
7.5x10^6
5.0x10^6
2.5x10^6
2.6x10^4
B16 F10 Melanoma Cells / Flask
Control
10 μg
25 μg
0
2
4
6
8
Days in Culture

FIG. 11F

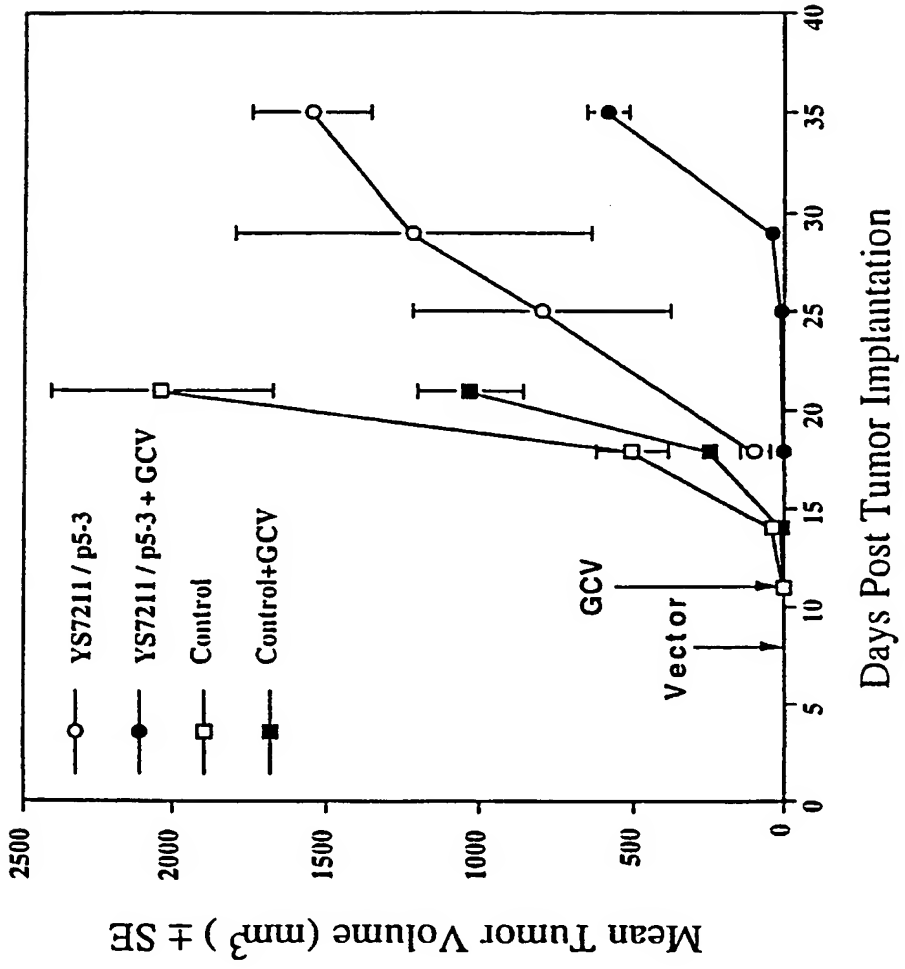
Mean Tumor Volume (mm³) ± SE
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
Days Post Tumor Implantation
YS7211 / p5-3
YS7211 / p5-3 + GCV
Control
Control+GCV
GCV
Vector

FIG.11G

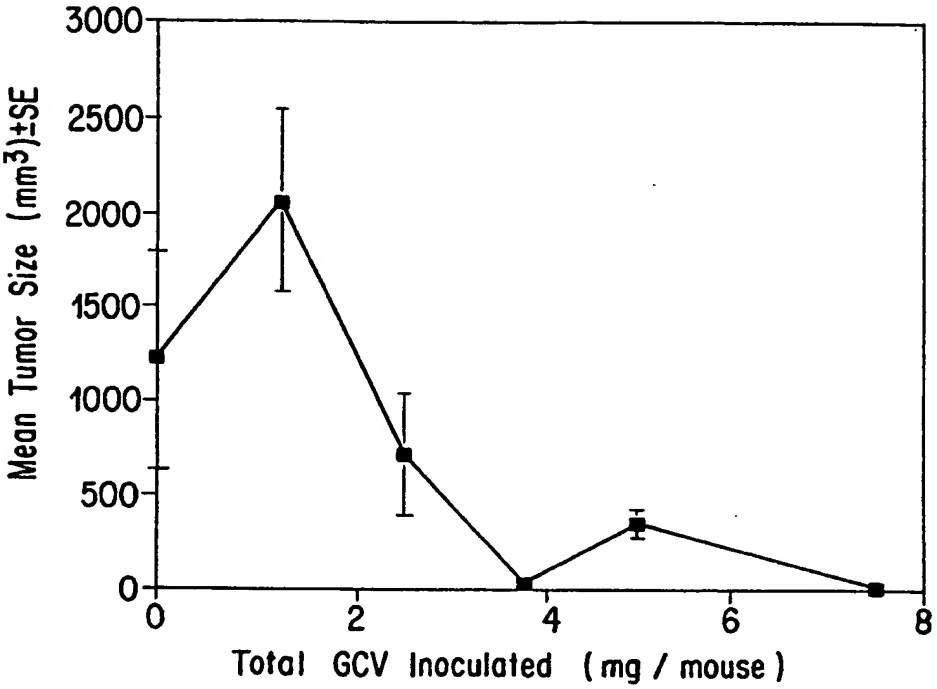
3000
2500
2000
1500
1000
500
0
Mean Tumor Size (mm3)±SE
0
2
4
6
8
Total GCV Inoculated (mg / mouse)

FIG. 11H

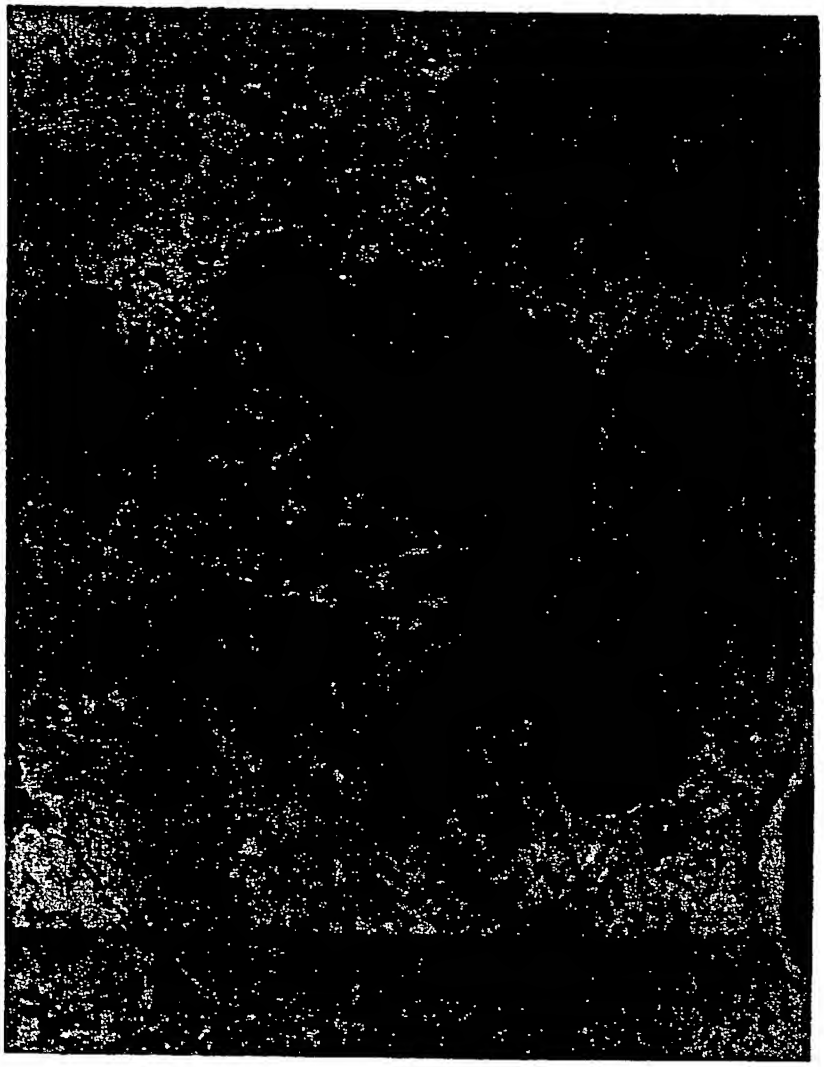

FIG. 12A

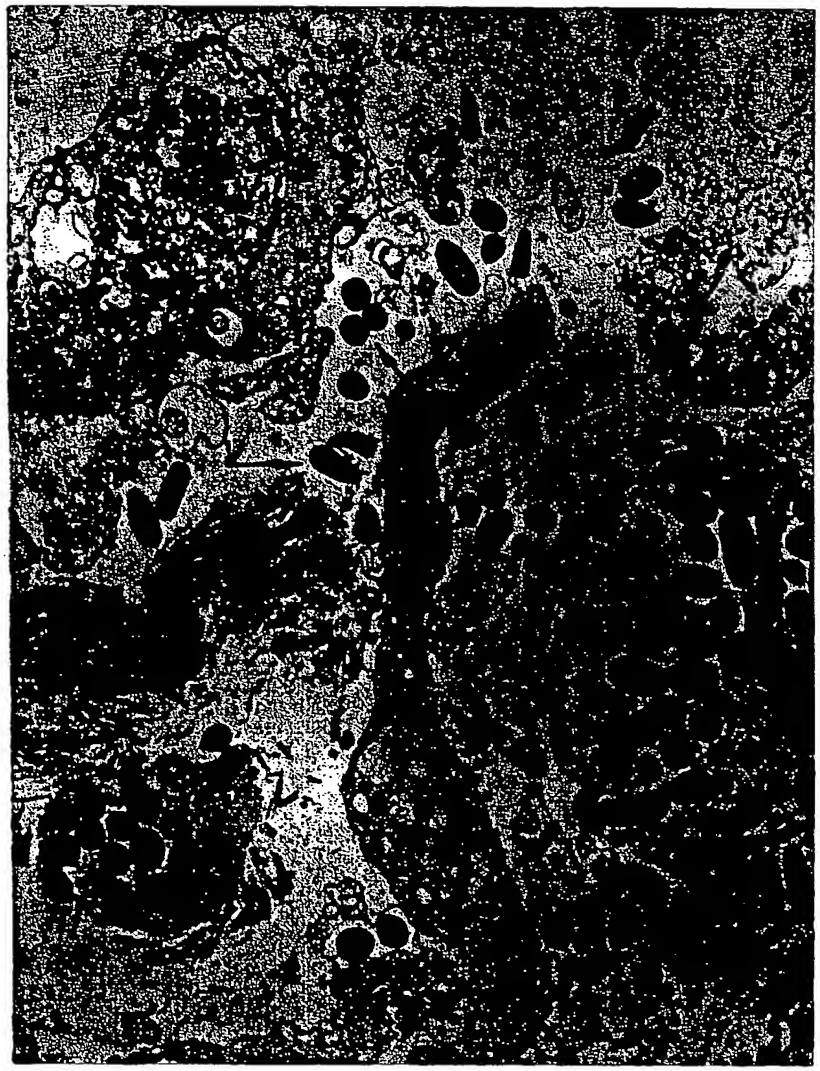

FIG. 12B

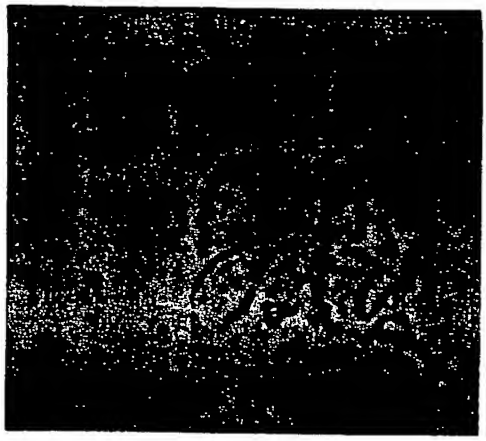

FIG. 13A

FIG. 13B

FIG.14A

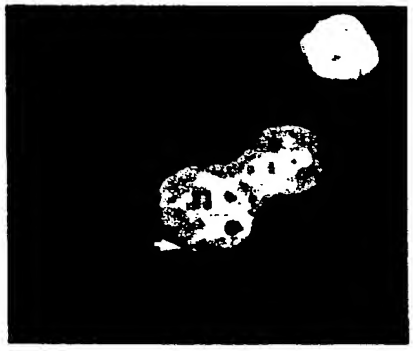

FIG.14B

FIG.14C

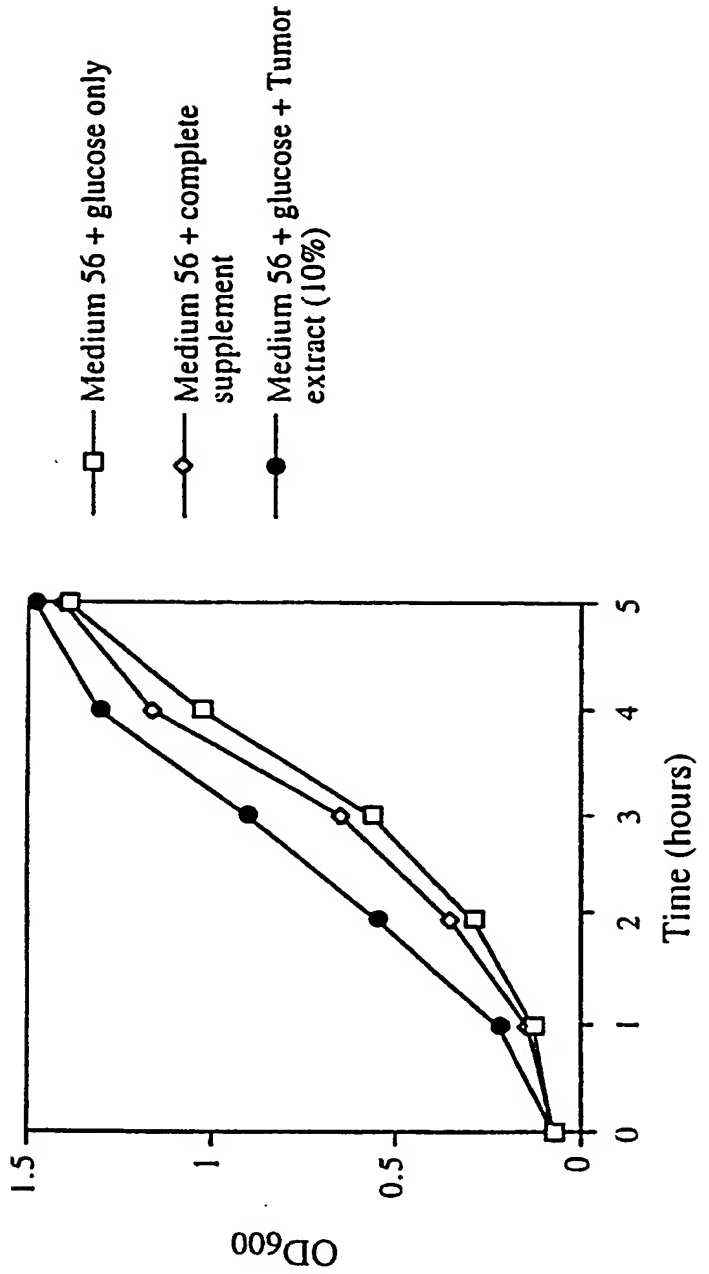
Medium 56 + glucose only
Medium 56 + complete supplement
Medium 56 + glucose + Tumor extract (10%)
OD600
1.5
1
0.5
0
0
1
2
3
4
5
Time (hours)

FIG. 15A

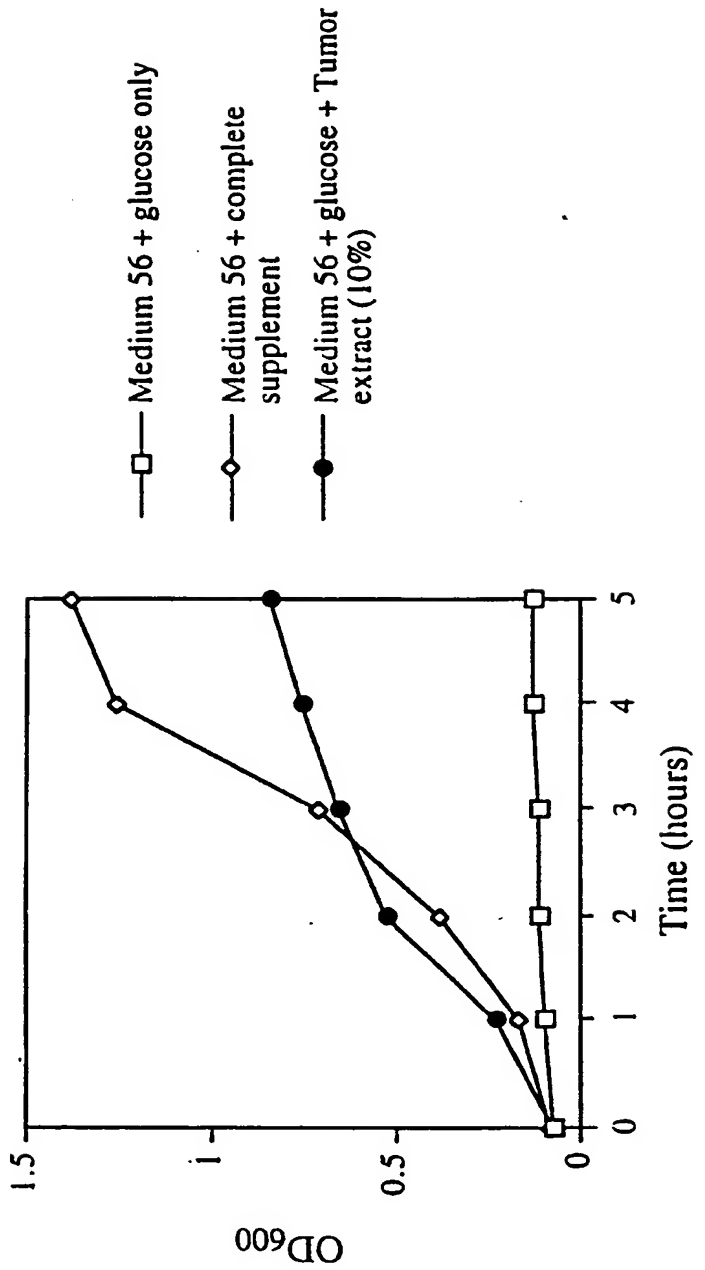
Medium 56 + glucose only
Medium 56 + complete supplement
Medium 56 + glucose + Tumor extract (10%)
OD600
1.5
1
0.5
0
0
1
2
3
4
5
Time (hours)

FIG.15B

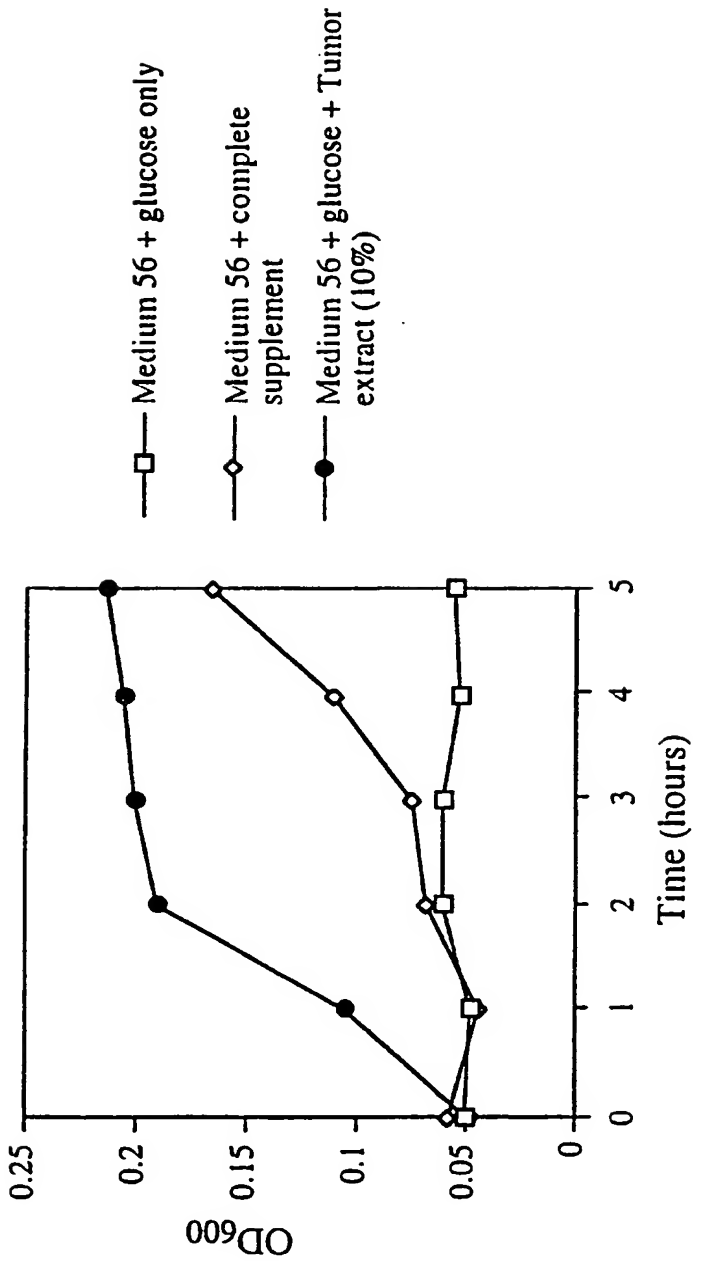
Medium 56 + glucose only
Medium 56 + complete supplement
Medium 56 + glucose + Tumor extract (10%)
OD600
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
Time (hours)

FIG. 15C

Growth of Clones 72 and YS7212 in Human M2 Melanoma Cultures

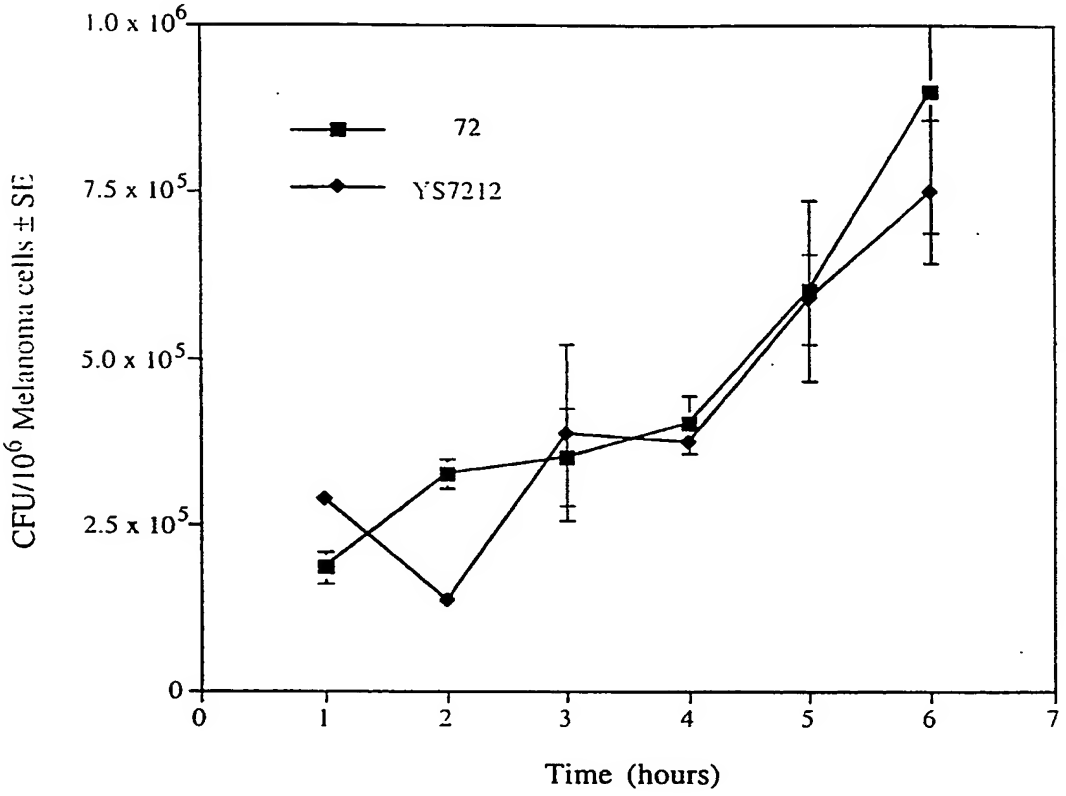

FIG. 15D

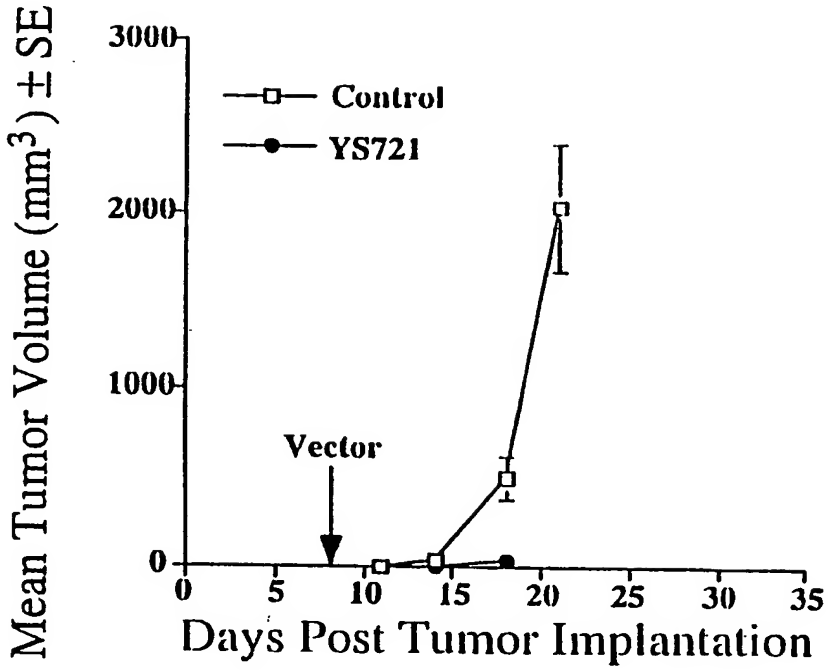

FIG. 16A

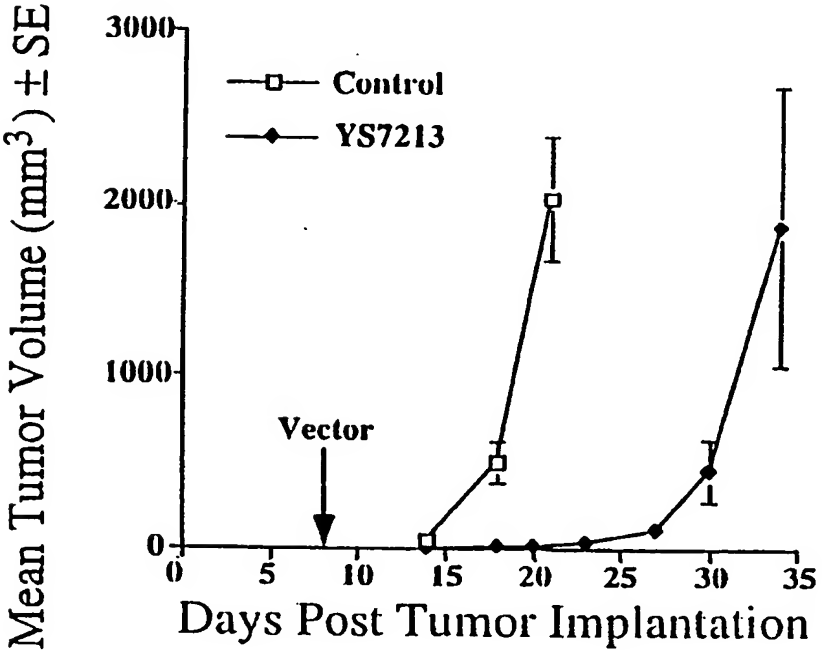

FIG. 16B

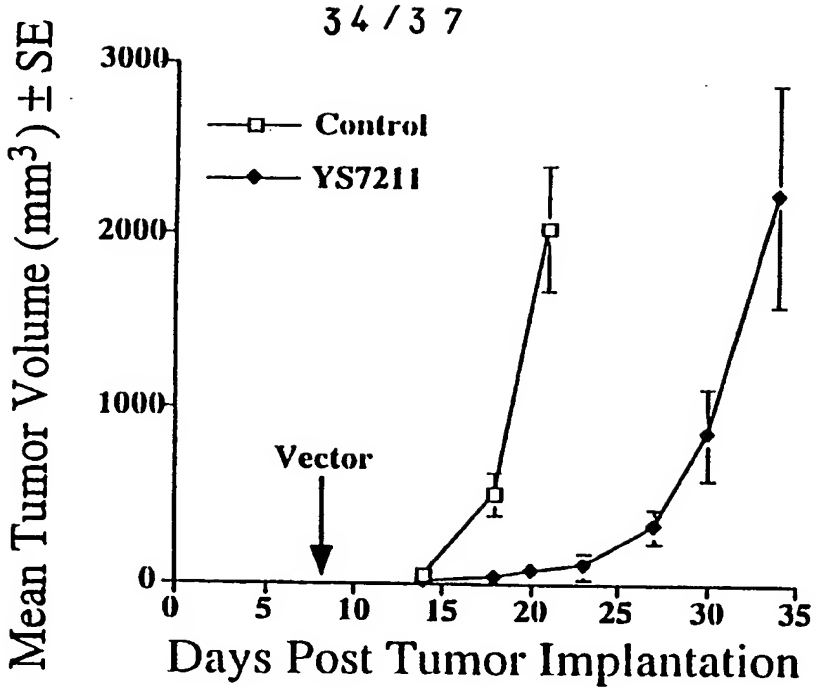

FIG. 16C

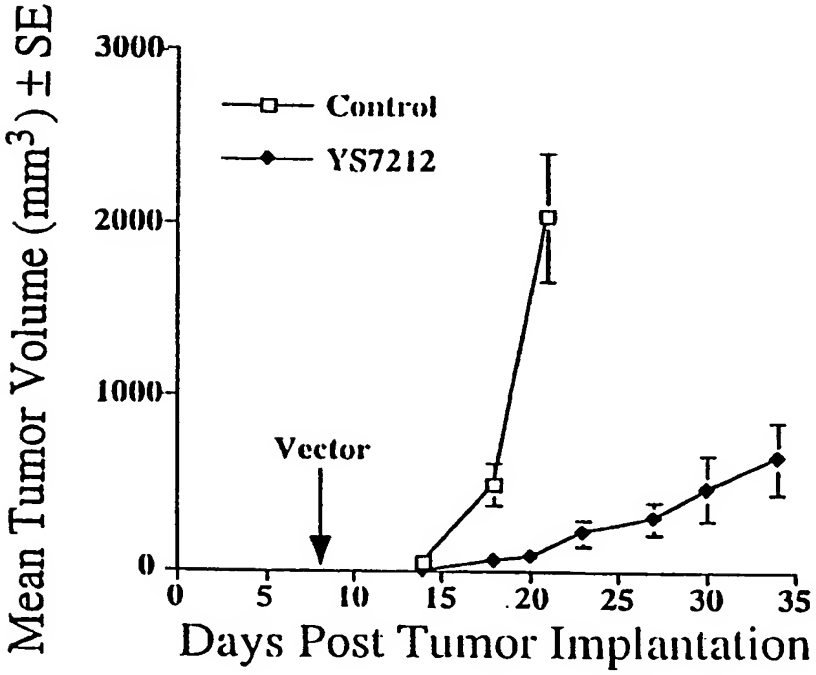

FIG. 16D

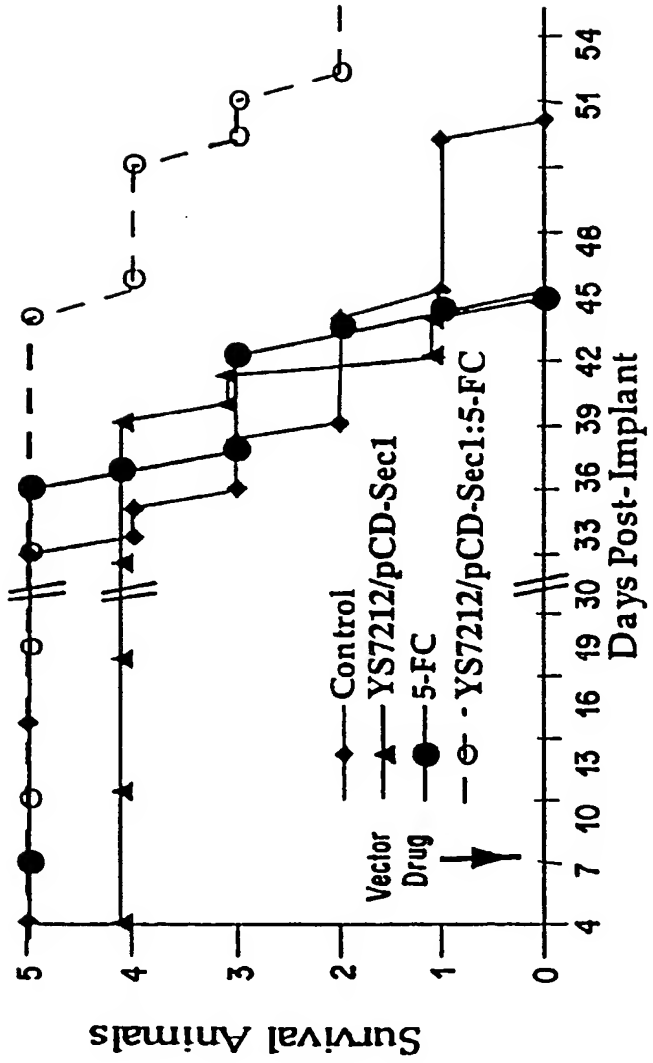
Survival Animals
5
4
3
2
1
0
4 7 10 13 16 19 30 33 36 39 42 45 48 51 54
Days Post-Implant
Vector
Drug
Control
YS7212/pCD-Sec1
5-FC
- YS7212/pCD-Sec1:5-FC

FIG.17

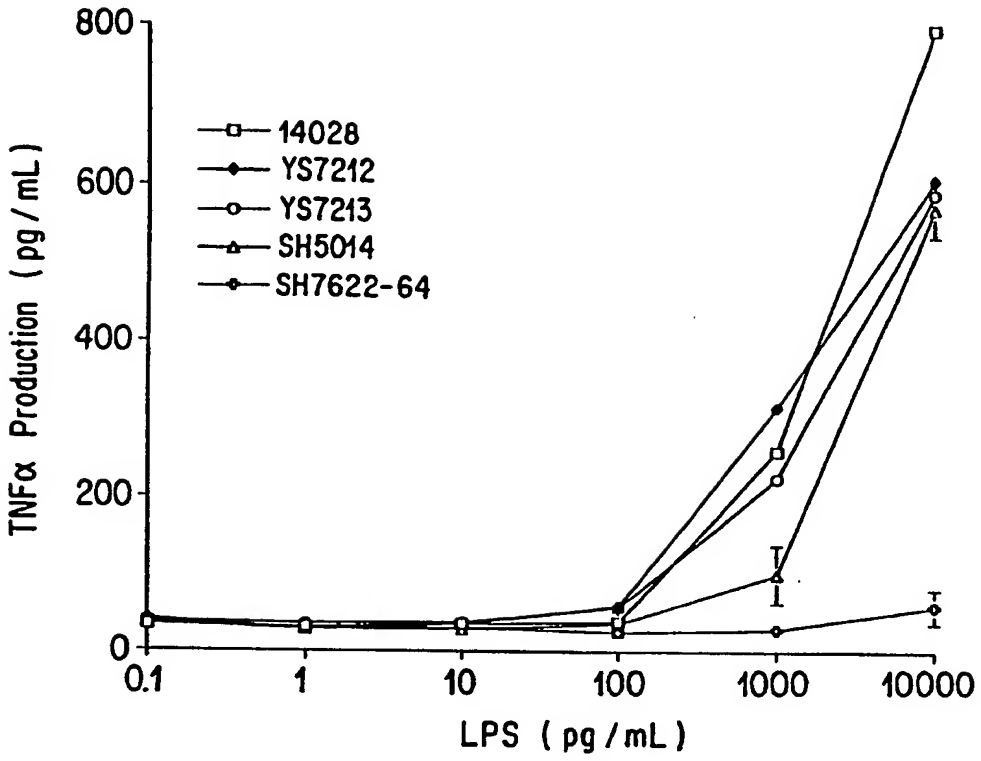
800
600
400
200
0
TNFα Production (pg/mL)
14028
YS7212
YS7213
SH5014
SH7622-64
0.1
1
10
100
1000
10000
LPS (pg/mL)

FIG. 18

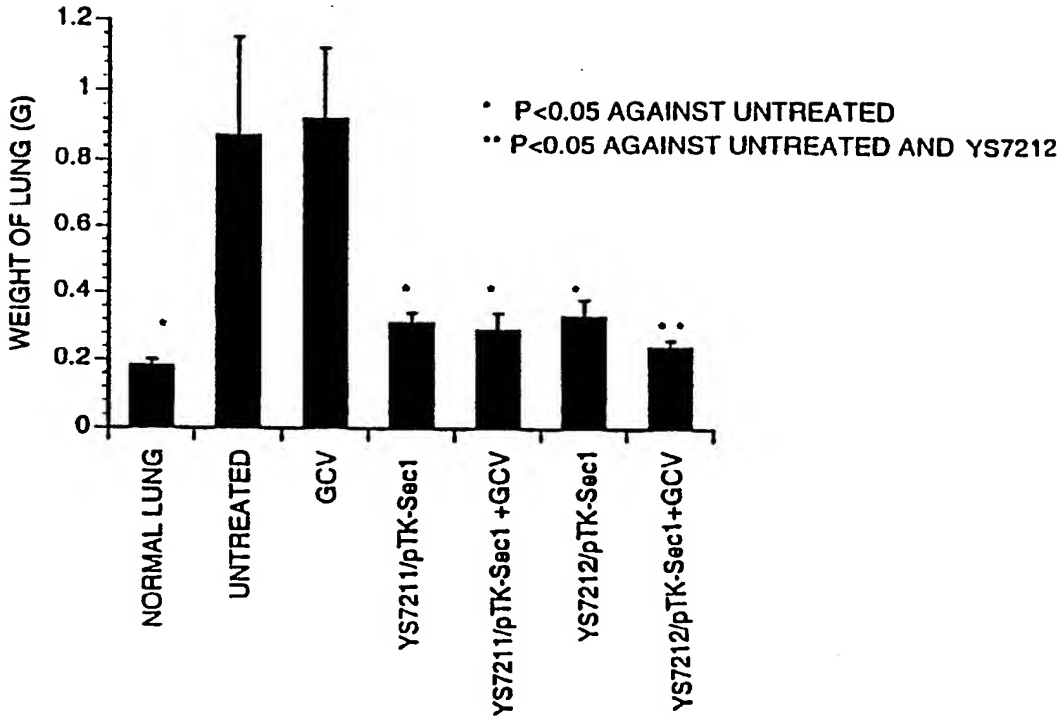
WEIGHT OF LUNG (G)
1.2
1
0.8
0.6
0.4
0.2
0
* P<0.05 AGAINST UNTREATED
** P<0.05 AGAINST UNTREATED AND YS7212
NORMAL LUNG
UNTREATED
GCV
YS7211/pTK-Sec1
YS7211/pTK-Sec1 +GCV
YS7212/pTK-Sec1
YS7212/pTK-Sec1+GCV

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10250

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/234.1, 258.1; 435/4, 6, 29, 252.1, 252.3, 252.8, 320.1, 879; 436/64; 530/351; 935/22, 27, 29, 72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT
search terms: salmonella?, typhimurium?, cancer?, tumor?, tumour?, treat?, administ?, diagnos?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MCLAUGHLIN et al. Synergistic Activity of Components of Mycobacteria and Mutant Salmonella in Causing Regression of Line-10 Tumors in Guinea Pigs. Cancer Research. May 1979, Vol. 39, No. 5, pages 1766-1771, see entire document.	1-65
A	US 4,436,727 A (E. E. RIBI) 13 March 1984, see entire document.	1-65
A	US 5,021,234 A (U. EHRENFELD) 04 June 1991, see entire document.	1-65
A	US 5,344,762 A (A. KARAPETIAN) 06 September 1994, see entire document.	1-65

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 AUGUST 1996	Date of mailing of the international search report 10 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. DEGEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10250

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10250

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/02, 39/112; C07K 14/525; C12N; 1/02, 15/63, 15/74; G01N 33/48

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/234.1, 258.1; 435/4, 6, 29, 252.1, 252.3, 252.8, 320.1, 879; 436/64; 530/351; 935/22, 27, 29, 72

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-4, partially, 5-43, and 57-65, partially, drawn to Salmonella typhimurium strains, plasmids carried by these strains, kits containing the bacteria and a method of treating cancer using the microorganisms.

Group II, claims 1-4, partially, 44-56, and 57-65, partially, drawn to Salmonella typhimurium strains, plasmids carried by these strains, kits containing the bacteria and a method of diagnosing cancer using the microorganisms.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Although the Salmonella strains are the same, they are used for two distinct purposes; the treatment of cancer and the diagnosis of cancer. These two methods are distinct because the ultimate goals are different and the steps used in carrying out these aims are also different. Note that PCT Rule 13 does not provide for multiple methods of use within a single application. (37 C. F. R. 1.475(d)).